PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. 07-1008-WO-US)

In re Application of:	)	
	)	
Lawson et al.	)	
	)	Group Art Unit: 1644
Serial No.: 10/578,384	)	
	)	Examiner: Phillip Gambel
Filing Date: January 16, 2007	)	
	)	Confirmation No.: 1913
For: Methods for the Treatment of Inflammatory Bowel Disease By Administration of an Inhibitor of CSF-1 Activity	)	

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

DECLARATION OF DIANE MARSHALL, PhD., UNDER 37 CFR 1.132

I, Diane Marshall, in support of the above-identified United States patent application, do declare and state as follows:

1. I and co-inventors Timothy Bourne and Alastair David G. Lawson are the first, original, and joint inventors of the subject matter claimed in United States Patent Application Serial No. 10/578,384, filed on January 16, 2007, and entitled "Methods for the Treatment of Inflammatory Bowel Disease By Administration of an Inhibitor of CSF-1 Activity," which claims priority benefit of International Application PCT/GB04/04652 filed November 3, 2004, which claims the benefit of Great Britain application 0325836.5 filed November 5, 2003. I am making this Declaration in support of this application.

2. I received a 2:1 Bachelor of Science degree with Honors in biochemistry from the University of Birmingham in 1990. I was awarded a PhD. degree from the University of London in 1997; my thesis was entitled "Investigation of clearance systems in antibody targeted therapy of cancer." I then held a post doctoral position within the Imperial College School of Medicine,

London, focusing on radiolabelled antibody targeting of inflammation, until 2001. In September 2001 I was employed by Celltech, now UCB, with a focus on the establishment of animal models of inflammatory bowel disease ("IBD"). I have directed and written UCB's Pharmacology strategy for IBD, highlighting the clinical disease pathology, limitations of current treatments for IBD, the novel therapeutics under investigation and the animal models available for pre-clinical assessment of new treatments for IBD. I am considered an expert in the field of IBD within the research organization of UCB, where my opinion is sought for IBD focused projects. I have designed the studies of UCB's novel therapeutics in UCB's animal models of IBD. I have attended international meetings in the field of IBD. Due to my knowledge in the area of IBD, I am now leading UCB's Disease Area Expert Group for Gastroenterology and Dermatology. A listing of my publications is attached hereto as Exhibit 1.

3. I am currently employed by UCB Pharma, S.A., the assignee of this patent application. My efforts in connection with the preparation of the Declaration are within the scope of my employment duties.

4. Inflammatory bowel disease (IBD) refers to serious, chronic disorders of the intestinal tract characterized by chronic inflammation at various sites in the gastrointestinal tract. Its cause is not known, although genetic and environmental factors are believed to contribute to susceptibility to IBD. Various attempts have been made to provide therapies for those suffering from IBD. Such attempts have included the use of steroids and non-steroidal anti-inflammatory drugs (NSAIDs) such as 5-aminosalicylic acid drugs. Such treatments can be problematic, in that these drugs may not be suitable for long-term use, and are not effective in some 30% of IBD patients. For these patients, attempted therapies have included immunosuppressive and immunoregulatory agents; in some cases surgery is required. In that regard, many different cytokines and T-lymphocyte cell types have been implicated in IBD, all of which present potential targets for IBD treatment.

5. Thus it is recognized in the art that IBD and its treatment are both unpredictable. It cannot be known which proposed treatments will be effective for particular patients. In particular, not all anti-inflammatories are effective in the treatment of IBD. Steroids and NSAIDS, although standard anti-inflammatories for a wide variety of inflammatory disorders, are not effective against IBD for a significant number of patients. Thus, the mere fact that a particular drug is an anti-inflammatory does not mean that it will be effective against IBD, even

though IBD is an inflammatory disorder. For some patients, no known treatments are effective. As of the filing date of this application, there is no known cure for IBD.

6. Colony stimulating factor 1 (hereinafter CSF-1), also known as macrophage colony stimulating factor (M-CSF) is a cytokine produced by a variety of cells, including macrophages, endothelial cells, and fibroblasts. While many different cytokines and T-lymphocyte cells have been implicated in IBD, it is not known if CSF-1 plays any role in the pathogenesis of IBD, although increased CSF-1 serum levels have been observed in patients with active IBD.

7. There are three known types of CSF's: CSF-1 (also known as M-CSF), G-CSF, and GM-CSF. Those skilled in the art understand that these different CSF's have different properties, and may not always be substituted for one another. These different properties have long been recognized by those skilled in the art, as shown in the following representative publications:

a) Falk, L.A. et al., "Analysis of Ia antigen expression in macrophages derived from bone marrow cells cultured in granulocyte-macrophage colony-stimulating factor or macrophage colony-stimulating factor," J. Immuno. 1988 April 15, 140(8) 2652-2660; erratum in J. Immunol. 1988 Jul 15; 141(2) 709 (Exhibit 2)

b) Willman, C.L. et al., "Regulation of MHC Class II Gene Expression in Macrophages by Hematopoietic Colony-Stimulating Factors (CSF), Induction by Granulocyte/Macrophage CSF and Inhibition by CSF-1," J. Exp. Med., 170, 1159-1567(1989) (Exhibit 3);

c) Falk, L.A. et al., "Differential Production of IFN-α/β by CSF-1 and GM-CSF-Derived Macrophages," J. Leukocyte Biology 48:43-49 (1990) (Exhibit 4);

d) Tadokoro, C.E. et al., "Bone marrow-derived macrophages grown in GM-CSF or M-CSF differ in their ability to produce IL-12 and to induce IFN-γ production after stimulation with *Trypanosoma cruzi* antigens," Immunology Letters, Vol. 77, issue 1, 1 May 2001, pages 31-38 (Exhibit 5);

e) Fleetwood, A.J. et al., "Granulocyte-Macrophage Colony-Stimulating Factor (CSF) and Macrophage CSF-Dependent Macrophage Phenotypes Display Differences in Cytokine Profiles and Transcription Factor activities: Implications for CSF Blockade in Inflammation," J. Immunol. 2007; 178; 5245-5252 (Exhibit 6).

8. The present invention relates to the discovery that anti-CSF-1 antibodies or fragments thereof have been shown to be effective therapeutically against IBD.

9. I have reviewed the Office Action mailed August 31, 2010 in this application, and the references cited therein, namely U.S. 7,108,852 to Devalaraja (hereinafter "Devalaraja"), U.S. 7,455,836 to Hamilton et al. (hereinafter "Hamilton"), U.S. 7,507,705 to Buschmann et al. (hereinafter "Buschmann"), and U.S. 2004/0053365 to Renner et al. (hereinafter "Renner"). Each of these references will be discussed in turn.

Devalaraja

10. Devalaraja states that the invention disclosed therein is connected to the discovery that CSFs appear to be critical for leukocyte recruitment, specifically polymorpho-nuclear neutrophil (PMN) and monocyte recruitment, and exhibit synergizing activity with chemokines (col. 4, lines 3-7). The CSF can be either M-CSF (col. 4, lines 38-39, 62-63), G-CSF (col. 5, lines 16-17, 40-41), or GM-CSF (col. 5, line 60). Devalaraja states that the diseases or disorders treated can include inflammation, osteoporosis, autoimmune disease, and atherosclerosis (col. 6, lines 1-8). Diseases specifically noted are atherosclerosis (col. 6, line 16), sepsis (col. 6, line 25), asthma (col. 6, line 33), autoimmune disease (col. 6, lines 41-42), osteoporosis (col. 6, line 51), rheumatoid arthritis (col. 6, lines 60-61), and osteoarthritis (col. 7, line 3).

11. At col. 7, lines 4-8, examples of autoimmune diseases are stated to include "SLE, GVHD, RA, IBD, asthma, and psoriasis."

12. In the definition of "autoimmune disease" at col. 11 lines 4-16, IBD is not listed as either "highly probable" or "probable." IBD is not included as one of the "preferred inflammatory diseases" listed at col. 11, lines 21-25. The claims of Devalaraja are limited to a method of treating rheumatoid arthritis, and do not recite any method of treating of IBD.

13. The data presented in Devalaraja include experiments conducted to confirm that G-CSF synergizes the IL-8 induced chemotaxis *in vivo* and *in vitro* (col. 18, line 38 – col., 19, line 17; Figs. 1-13) and experiments conducted to demonstrate that M-CSF synergized MCP-1 induced chemotaxis (col. 19, lines 18-62; Figs. 14-21). Of these, the data related directly to M-CSF is presented at Figs. 19-21. Devalaraja does not include any animal model data for IBD, nor

does it contain any animal model data for any of the diseases stated to be treatable by the method disclosed therein.

14. As one skilled in the art, to me the Devalaraja reference does not disclose a treatment of IBD by administration of anti-M-CSF antibodies. I base this understanding on the fact that IBD is not included in the list of individual diseases at column 6, nor is it explicitly included in the definition of "autoimmune disease" at col. 11, lines 4-16, and a method of treating IBD is not recited in the claims. The only time IBD is mentioned is at col. 7, lines 4-8, but that statement is with respect to "preferred use of inhibitors of the present invention" and does not specifically mention anti-M-CSF antibodies. The patent does not limit such "inhibitors" to anti-M-CSF antibodies, but also includes data for anti-G-CSF antibodies and anti-GM-CSF antibodies as inhibitors. One skilled in the art would not understand from the sentence at col. 7, lines 4-8 which antibodies Devalaraja intended to suggest for the treatment of IBD. Also, the absence of any experiments using an animal model for IBD indicates to me that Devalaraja did not demonstrate that anti-M-CSF can be effective in the treatment of IBD. Given the well-known lack of predictability of the effectiveness of IBD treatments and the fact that not all anti-inflammatory treatments are effective against IBD, the paucity of discussion of IBD in the Devalaraja disclosure suggests to me as one skilled in the art that Devalaraja was not in possession of a method of treating IBD using an anti-M-CSF antibody or antibody fragment. As one skilled in the art, I would not have combined the teachings of Devalaraja with my own knowledge to understand that IBD could be treated by the administration of anti-M-CSF antibodies.

Hamilton

15. The Hamilton reference does not teach me as one skilled in the art that Hamilton was in possession of a method of treating IBD by administering anti-M-CSF antibodies. Hamilton discloses that the term "ameliorate" as used therein refers to the reduction of the symptoms exhibited in patients suffering from an inflammatory condition, including the more specific response of reducing levels of inflammatory and pro-inflammatory mediators such an M-CSF, GM-CSF, IL-1, TNF-α, IL-6, products of COX-s, u-PA and other molecules (col. 5, lines 54-62). The only data presented for anti-M-CSF antibodies is at Figure 2C, and relates to the treatment of collagen-induced-arthritis (CIA). The only animal models presented are for CIA, asthma, and COPD. The claims are limited to the use of anti-GM-CSF antibodies. The statement at column 5, lines 44-50 that "inflammatory condition" relates to chronic inflammation

conditions such as rheumatoid arthritis, inflammatory bowel disease, Crohn's disease, type I diabetes, multiple sclerosis, psoriasis ad chronic inflammatory lung disease such as asthma, chronic bronchitis, emphysema or chronic obstructive airway disease does not suggest to me that Hamilton was in possession of a method of treatment of either inflammatory bowel disease or Crohn's disease, given the unpredictable nature of these disorders and methods of treatments thereof. The fact that anti-M-CSF antibody was shown to have some effect in animal models in the treatment of collagen-induced-arthritis teaches nothing to one skilled in the art about possible treatments for IBD. Similarly, the data relating to anti-GM-CSF antibodies does not teach one of skill in the art about the effects of anti-M-CSF antibodies, either on the disorders for which anti-GM-CSF antibodies were used, or for any other disorders. As one skilled in the art, I would not have combined the teachings of Hamilton with my own knowledge to understand that IBD could be treated by the administration of anti-M-CSF antibodies.

Renner

16. Renner relates to humanized GM-CSF antibodies. As one skilled in the art, this disclosure teaches me nothing about the properties or effects of anti-M-CSF antibodies. Renner discloses at paragraph [0004] that GM-CSF is known to play a role in the development of rheumatoid arthritis. As one skilled in the art, this teaches nothing about what factors may play a role in the development of other inflammatory diseases. Paragraph [0040] recites "inflammatory conditions" including both specific and non-specific immune reaction to an antigen. The fact that IBD is included in this list does not teach me as one skilled in the art that anti-GM-CSF antibodies would be effective in the treatment of IBD, and even if that were the teaching, it does not teach anything about the effect of anti-M-CSF antibodies in the treatment of IBD. Renner does not claim a method for treatment of IBD, or a method of treatment of any disorder.

Buschmann

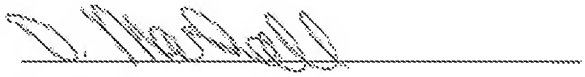
17. Buschmann is directed to the use of colony stimulating factor (CSF) or a nucleic acid molecule encoding CSF for preparation of a pharmaceutical composition for enhancing neovascularization and/or the growth of collateral arteries and/or other arteries from preexisting arteriolar connections (col. 5, lines 5-10); Buschmann also is directed to treatment of tumors by use of an agent which suppresses neovascularization or growth of collateral arteries through the inhibition of the biological activity of a CSF (col. 8, line 66 - col. 9, line 4). The agent can be an anti-CSF antibody (col. 10, line 65 – col. 11, line 2) but Buschmann does not distinguish between the different types of CSF's in this regard. Buschmann has nothing to do with inflammatory

disorders, or the treatment of inflammatory disorders. As one skilled in the art, this disclosure does not teach me that these different CSF's or their antibodies are equivalent with respect to the treatment of any inflammatory disease, and does not teach anything about treatment of IBD.

18. As one skilled in the art, I see no motivation to combine Devalajara with Hamilton, nor would doing so lead one skilled in the art to use anti-M-CSF antibodies in the treatment of IBD. Nor would these references have led me to believe that the use of anti-M-CSF antibodies in the treatment of IBD would have had a reasonable chance of success. The biological and pharmaceutical arts are generally unpredictable, and IBD in particular is unpredictable and notoriously difficult to treat. I note that the Devalaraja and Hamilton references were each published in 2002, Renner was published in 2004, and Buschmann was published in 1999. There has long been a need for an effective treatment for IBD. The fact that none of the references makes such a claim suggests to me that none of these presumptively skilled workers recognized that such a treatment could work. The only way to determine that such a treatment could work is through the use of animal modeling studies such as are reported in the present application.

19. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this Declaration, the patent application, or any patents issuing thereon.

Declared this 8th day of February 2011,



Diane Marshall, Ph.D.

EXHIBIT 1

Diane Marshall, PhD., List of Publications

PAPERS

Kinnear G, Wood KJ, Marshall D, Jones ND. 2010. Anti-OX40 prevents effector T-cell accumulation and CD8+ T-cell mediated skin allograft rejection. Transplantation 90 (12) 1265-1271.

Eddleston A, Marenzana M, Moore AR, Stephens P, Muzylak M, Marshall D, Robinson MK. 2009. A short treatment with an antibody to sclerostin can inhibit bone loss in an ongoing model of colitis. *J. Bone Miner. Res. 24 (10) 1662-1671.*

Marshall D, Cameron J, Lightwood D, Lawson AD. 2007. Blockade of colony stimulating factor-1 (CSF-1) leads to inhibition of DSS-induced colitis. *Inflamm. Bowel Dis. 13(2): 219-24.*

Wei S, Lightwood D, Ladyman H, Cross S, Neale H, Griffiths M, Adams R, Marshall D, Lawson A, McKnight AJ, Stanley ER. 2005. Modulation of CSF-1-regulated post-natal development with anti-CSF-1 antibody. *Immunobiology 210 (2-4): 109-119.*

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Larbi KY, Dangerfield JP, Culley FJ, Marshall D, Haskard DO, Jose PJ, Williams TJ, Nourshargh S. 2003. P-selectin mediates IL-13-induced eosinophil transmigration but not eotaxin generation in vivo: a comparative study with IL-4-elicited responses. *J. Leukoc. Biol. 73: 65-73.*

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McHale JF, Harari OA, Marshall D, and Haskard DO. 1999. TNF-α and IL-1 sequentially induce endothelial ICAM-1 and VCAM-1 expression in MRL/*lpr* lupus-prone mice. *J. Immunol. 163: 3993-4000.*

McHale JF, Harari OA, Marshall D, and Haskard DO. 1999. Vascular endothelial cell expression of ICAM-1 and VCAM-1 at the onset of eliciting contact hypersensitivity in mice: evidence for a dominant role of TNF-α. *J. Immunol. 162: 1648-1655.*

Al-Mufti RAM, Pedley RB, Marshall D, Begent RHJ, Hilson A, Winslet MC and Hobbs KEF. 1999. In vitro assessment of Lipiodol-targeted radiotherapy for liver and colorectal cancer cell lines. *Br. J. Cancer 79: 1665-1671.*

Springer CJ, Bavetsias V, Jackman AL, Boyle FT, Marshall D, Pedley RB and Bisset GMF. 1996. Prodrugs of thymidylate synthase inhibitors: potential for antibody directed enzyme prodrug therapy (ADEPT). *Anti-Cancer Drug Design 11: 625-636.*

Marshall D, Pedley RB, Boden JA, Boden R, Melton RG and Begent RHJ. 1996. Polyethylene glycol modification of a galactosylated streptavidin clearing agent: effects on immunogenicity and clearance of a biotinylated intact anti-tumour antibody. *Br. J. Cancer 73: 565-572.*

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POSTERS

Kinnear GJ, Vugler A, Marshall D, Moore A, Johnson Z, Shaw S. 2008. Determining the cytokine dependency of the acute phase response in CFA. *Rheumatology 47, Suppl 2, 61.*

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Marshall D, Cameron J, Lightwood D, Lawson ADG, Foulkes R. 2005. An antibody to colony stimulating factor-1 (CSF-1) inhibits DSS-induced colitis. Proceedings of the British Pharmacological Society at http://www.pa2online.org/abstracts/Vol3Issue4abst081P.pdf

Marshall D, Akhtar R, Cameron JD, Moore AR, Gozzard N, Foulkes R. 2003. Antibodies to the leukocyte integrin molecules α4 and LFA-1 inhibit DSS-induced colitis in rats. P018, The British Pharmacological Society Winter meeting 2002, Brighton. *British Journal Pharmacol. 138, April, 88P.*

Marshall D, Bhatia VK, Ahmed S, Lidington EA, Larbi KY, Nourshargh S, Haskard DO. 2001. Expression of P- but not E-selectin in peritoneal microvasculature during inflammatory peritonitis. *The FASEB Journal 15:A328.*

Larbi KY, Dangerfield JP, Thompson RD, Marshall D, Haskard DO, Nourshargh S. 2000. P-selectin is required for IL-4-induced eosinophil migration into the mouse peritoneal cavity but not into the mouse cremaster muscle. *The FASEB Journal 14:A706.*

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Marshall D, Pedley RB, Boden JA, Boden R and Begent RHJ. 1993. Improving radioimmunotargeting using streptavidin for rapid clearance of circulating radiolabilled biotinylated antibodies. *Br. J. Cancer 67: Suppl XX, 70.*

EXHIBIT 2

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ANALYSIS OF Ia ANTIGEN EXPRESSION IN MACROPHAGES DERIVED FROM BONE MARROW CELLS CULTURED IN GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR OR MACROPHAGE COLONY-STIMULATING FACTOR[1]

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Bone marrow cells from C3H/HeJ mice were cultured in recombinant granulocyte-macrophage-CSF (rGM-CSF) or recombinant or purified (natural) preparations of macrophage-CSF (CSF-1) for 7 days, the adherent macrophages removed enzymatically, recultured in the absence of growth factor, and examined for their differentiative and functional characteristics. Expression of Ia Ag differed markedly in these two populations. Antibody plus complement-mediated cytotoxicity indicated that the percent of Ia-positive macrophages was low (~15 to 20%) in both populations. Treatment of cultures with rIFN-γ increased the percentage of Ia-positive cells in both populations; however, more CSF-1-derived macrophages were induced to become Ia positive than rGM-CSF-derived macrophages. In contrast, total Ia expression and Ia density per cell, as measured by ELISA and quantitative immunofluorescence, respectively, showed that medium-treated rGM-CSF-derived macrophages exhibited greater total Ia expression and higher Ia density per cell than CSF-1-derived macrophages. Treatment of CSF-1-derived macrophages with rIFN-γ increased total Ia expression per culture to the levels exhibited by rGM-CSF-derived cells, although the density of Ia Ag per cell remained lower than in rGM-CSF-derived cells (medium or rIFN-γ-treated). Northern blot and slot blot analysis of cytoplasmic RNA extracted from freshly harvested rGM-CSF- or CSF-1-derived cells indicated that the differences seen in basal Ia expression were also reflected at the level of steady-state, Ia-specific mRNA.

The functional capacity of these two macrophage populations to stimulate Ag-specific T cell proliferation was also assessed. rGM-CSF- or CSF-1-derived macrophages were first cultured in the absence or presence of rIFN-γ, Ag-pulsed, and irradiated before co-culture with Ag-primed, purified T cells. Ag-induced T cell proliferation was significantly greater in rGM-CSF- than in CSF-1-derived macrophages. Treatment of either population with rIFN-γ had only a miminal effect on the ability of either macrophage population to stimulate Ag-specific T cell proliferation. These findings suggest that the development of mature macrophages from bone marrow progenitors under the influence of either GM-CSF or CSF-1 may, in part, underlie the functional heterogeneity of different macrophage populations.

Hemopoietic cells of bone marrow origin proliferate and differentiate into more mature forms under the influence of a family of glycoproteins which are referred to collectively as CSF (1, 2). The macrophage is one such cell type, and bone marrow-derived macrophage progenitors have been shown to develop along the monocytic lineage under the influence of several different types of CSF: CSF-1,[4] GM-CSF, and, to a more limited extent, IL-3 (reviewed in Refs. 2 and 3).

As macrophages differentiate into mature effector cells, they acquire a series of phenotypic "markers" which are essential to specific effector functions and are associated with various stages of differentiation (3, 4). For example, the development of isotype-specific FcR facilitates the uptake of particles opsonized with antibody or antibody plus C (5). Another differentiation marker, the class II MHC Ag (i.e., Ia Ag), are expressed by macrophages and are necessary for the appropriate presentation of processed Ag to T cells which bear specific AgR in the context of a specific Ia molecule (6–8). This extremely specific interaction between the APC and the T cell triggers a series of events which lead to the activation and expansion of the Ag-specific T cell (6, 7).

Within the past decade, the study of Ia Ag expression on the surface of macrophages has focused principally on its inducibility by IFN-γ in populations of macrophages which are primarily Ia negative (9–12). However, early work by Cowing et al. (13) demonstrated that macrophages obtained from different organs varied greatly in their basal expression of Ia Ag. For instance, resident

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[1] This work was supported by National Institutes of Health Grant AI-18797. The opinions or assertions contained herein are the private views of the authors and should not be construed as official or necessarily reflecting the views of the Uniformed Services University of the Health Sciences or the Department of Defense. The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council DHEW Publication No. (NIH) 85-23.
[2] This paper represents work done in partial fulfillment of the requirements for the Ph.D. degree.
[3] To whom correspondence and reprint requests should be directed.
[4] Abbreviations used in this paper: CSF-1, macrophage CSF; GM-CSF, granulocyte-macrophage CSF; KLH, keyhole limpet hemocyanin; EMEM, Eagle's MEM; EBSS, Earle's balanced salt solution.

peritoneal macrophages were found to express very low levels of Ia Ag, whereas splenic macrophages possessed constitutively high expression, even in athymic nude mice where the production of IFN-γ by T cells would be expected to be limited [14] and would have to be provided compensatorily from non-T cell sources [15]. This suggests that mechanisms in addition to IFN-γ induction may exist to explain the selective expression of Ia Ag on specific macrophage populations during development. One possibility is that different CSF give rise to macrophage populations which differ with respect to Ia Ag expression and associated functions.

In this report, we have examined the expression and function of Ia Ag on macrophages derived from bone marrow progenitors in the presence of rGM-CSF vs those derived from bone marrow progenitors under the influence of recombinant or highly purified preparations of CSF-1. Our results demonstrate that bone marrow-derived macrophages which develop under the influence of CSF-1 exhibit low basal levels of Ia Ag expression and Ia-specific mRNA, and exhibit a reduced capacity to stimulate Ag-specific T cell proliferation when compared with macrophages which were derived under the influence of rGM-CSF. Both the expression of Ia, and to a lesser extent, the ability to induce Ag-specific T cell proliferation, could be augmented in CSF-1-derived macrophages by treatment with rIFN-γ. These findings suggest that the ability to generate heterogeneous populations of macrophages, based on the expression of specific differentiative markers and their associated functions, may be the result of the selective action of individual CSF on the bone marrow progenitors.

MATERIALS AND METHODS

Reagents. Murine CSF-1 was purified exactly as described elsewhere [16]. Briefly, L929 fibroblasts were grown to confluency in the presence of EMEM supplemented with 10% FCS, 2 mM glutamine, 15 mM HEPES, 0.02% sodium bicarbonate, penicillin/streptomycin (100 IU/ml and 100 mg/ml, respectively), and 50 μg/ml gentamicin at 37° C in 5% CO_2. Upon confluency, serum-containing medium was removed, the cells washed three times with EBSS and were cultured for an additional 7 days in serum-free medium. After this period of serum deprivation, supernatants were harvested and concentrated, and were subjected to hydroxylapatite column chromatography. Active fractions were eluted and applied to a DEAE Sephacel column and CSF-1 activity was eluted following a two-step, phosphate-NaCl gradient. This material was then applied to an Affi-Gel 202 column, which has been shown previously to retain IFN-α/β while allowing the CSF-1 activity to pass through the column. The CSF-1 activity was recovered and was purified by C-18 reverse-phase HPLC. The CSF-1 activity was eluted with an acetonitrile/trifluoroacetic acid linear gradient.

Murine rGM-CSF was the generous gift of Dr. John DeLamarter, Biogen, SA (Geneva, Switzerland). Human rCSF-1 was the kind gift of Cetus Corp. (Emeryville, CA). Murine rIFN-γ was the generous gift of Genentech, Inc. (South San Francisco, CA).

Bone marrow culturing techniques. Bone marrow progenitors were cultured in liquid culture in the presence of rGM-CSF or CSF-1 precisely as described previously [16]. Briefly, bone marrow cells were obtained from C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME) and were centrifuged on lymphocyte separation medium (Litton Bionetics, Charleston SC). Cells harvested from the interface were cultured at 1×10^5 cells/flask (for CSF-1) or 5×10^5 cells/flask (for rGM-CSF) in EMEM-10% FCS which contained 250 U/ml of the appropriate CSF (day 0). The concentration of CSF used, as well as the differences in the number of bone marrow cells seeded on day 0, were established in our previous study to minimize differences in total macrophage yield at the end of the 7-day culture period [16]. After a 24-h adherence step (day 1) which allowed for the removal of mature monocytes and fibroblasts from the bone marrow, nonadherent cells from each flask were transferred to a second flask and were supplemented with medium containing CSF (250 U/ml). The cells were again supplemented with CSF (250 U/ml) on day 4. After a total of 7 days in culture (day 7), macrophages were removed enzymatically with the neutral protease, Dispase II (Boehringer-Mannheim, Indianapolis, IN) and gentle scraping. The cells were collected and resuspended in RPMI 1640 (M.A. Bioproducts, Walkersville, MD), supplemented with 2% FCS and other additives as described above for EMEM-10% FCS (with the exception of gentamicin). Our previous studies have shown that the cells derived in this fashion in either CSF preparation are 100% mononuclear [16]. The rGM-CSF- or CSF-1-derived macrophages are recultured (as described below) at precise cell densities in the absence of exogenous CSF and equality of cell number is maintained in culture as assessed by Lowry [17].

Measurement of Ia Ag expression. Ia Ag expression was measured by three methods: direct complement-mediated cytotoxicity [12], ELISA [12], and by fluorescent antibody analysis through the use of the ACAS 470 [18] (described below). Direct complement-mediated cytotoxicity provides a measure of the percentage of Ia-positive macrophages. ELISA provides a relative measure of total Ia Ag expression per culture without regard for the number of Ia-positive cells. Fluorescent antibody analysis provided by the ACAS 470 provides a direct measure of Ia Ag density per cell. For all three assays, rGM-CSF- or CSF-1-derived macrophages were removed enzymatically on day 7 and recultured in the absence of CSF at 2×10^5 cells/well (for the direct cytotoxicity assay and ELISA) or at 1×10^5 cells/well (for fluorescent antibody analysis). Cultures were treated with medium only or medium plus rIFN-γ (5.0 U/ml) and examined at 24, 48, and 72 h after treatment. Cell density of medium-treated and IFN-γ-treated cultures was verified by examination of total protein as determined by the method of Lowry [17] and remained constant over the 3-day culture period with no loss in cell viability, as assessed by trypan blue exclusion.

In the cytotoxicity assay [12], the cells were first treated with affinity-purified, mAb anti-Ia^s [10-2.16 hybridoma; American Type Culture Collection, Rockville, MD] [5] and incubated for 45 min at 4° C. Subsequently, rabbit C (Low-Tox; Cedarlane Laboratories, Hornby, Ontario, Canada; diluted 1/12 in RPMI-2% FCS) was added to the monolayer. After a 45-min incubation at 37° C, trypan blue was added and the number of Ia-positive cells determined by counting the number of trypan blue-positive cells. The percentage of Ia-positive cells was determined after subtraction of the percentage of trypan blue-positive cells observed in cultures treated with C alone. C control cultures contained 3 to 4% trypan blue-positive cells and also provided a measure of the viability of the cells after 3 days in culture. In addition, cultures were also examined after treatment with an irrelevant mAb (anti-Ia^a) plus C. These cultures consistently exhibited ≤5% trypan blue-positive cells.

For ELISA [12], macrophage monolayers were fixed with 1% paraformaldehyde in PBS for 20 min at room temperature. After fixation, cells were washed extensively with EBSS-1% FCS and were then incubated with the same preparation of mAb anti-Ia^s or an irrelevant anti-Ia^a antibody for 45 min at 4° C. Cells were then washed and incubated with a peroxidase-conjugated secondary antibody (goat anti-mouse IgG,F(ab')$_2$; Cooper Biomedical, Inc., Malvern, PA) for 45 min at room temperature, followed by washing. The amount of enzyme-conjugated antibody that remained was measured by the addition of the substrate orthophenylenediamine and the incubation of cultures in the dark for 30 min at room temperature. The enzymatic reaction was stopped with the addition of 8 N sulfuric acid and the absorbance read at 490 nm (Bio-Tek EIA Reader, Burlington, VT). Absorbance (OD_{490}) observed in cultures treated with the irrelevant antibody was <0.09.

Ia density per cell was visualized by fluorescent antibody analysis through the use of the ACAS 470 [18] (Meridian Instruments Inc., Okemos, MI). Briefly, macrophage monolayers were treated as described above for Ia detection by ELISA except that in lieu of a peroxidase-conjugated secondary antibody, cultures were incubated with a fluorescein-conjugated secondary antibody (goat anti-mouse IgG F(ab')$_2$; Cooper Biomedical) for 45 min at 4° C in the dark. The following ACAS 470 parameters were utilized: wavelength = 488 nm, dichroic filter = 510 nm, step size = 2.0 μ, laser power = 200 mW, and scan strength = 10%. Background fluorescence was determined by treatment of macrophage monolayers with fluorescein-conjugated secondary antibody only.

Detection and quantification of Ia-specific cytoplasmic RNA. Ia-specific mRNA was isolated and examined according to the procedure of Fertsch et al. [19]. Briefly, 6×10^8 bone marrow-derived macrophages were removed enzymatically after 7 days in culture with rGM-CSF or CSF-1. The cells were washed three times with ice-cold EBSS, the cell pellet resuspended in an ice-cold isotonic solution. The cells were then lysed and the nuclei cleared from the lysate by centrifugation. The resulting cytoplasmic supernatant was subjected to a series of phenol and chloroform: phenol extractions to isolate

cytoplasmic RNA. The RNA was ethanol-precipitated overnight and the amount of RNA isolated was quantitated by measurement of absorbance at 260 nm. The RNA concentration was also verified by ethidium bromide staining of Northern blots. After precipitation, RNA preparations were examined by Northern blot and by slot blot analysis. For Northern blot analysis, RNA was denatured, electrophoresed on a 1% agarose-formaldehyde gel, and transferred to nitrocellulose. For slot blot analysis, RNA was denatured and applied to nitrocellulose filters with a Minifold Slot Manifold (Schleicher and Schueller, Keene, NH). Nitrocellulose filters were baked at 80° C for 2 h and then prehybridized overnight at 42° C. Filters were hybridized with 2×10^6 cpm/ml of ^{32}P-CTP-labeled probe (A^k_α) as described in detail by Fertsch et al. (19). Specific activity = ~10^9 cpm/μg) at 42° C for 18–24 h. After hybridization, blots were washed extensively, and were exposed to Kodak-XAR film at −70° C with intensifying screens. A densitometer (Hoefer Scientific Instruments, San Francisco, CA) was used to scan the autoradiograms and the peak areas from the recorded scans were calculated with a digitizer (Hewlett-Packard, Co., Fort Collin, CO) as described elsewhere (19). CRA cells (a constitutively Ia-positive, macrophage cell line) were the kind gift of Dr. Carol L. Reinisch (Tufts University School of Veterinary Medicine, Boston, MA) and served as a source of constitutive Ia message and, hence, was included as a positive control for Ia mRNA detection (19).

Measurement of Ag-induced T cell proliferation by rGM-CSF- and CSF-1-derived macrophages. The ability of Ag-pulsed rGM-CSF- and CSF-1-derived macrophages to induce Ag-specific induction of T cell proliferation was examined by a modification of the procedure of Lee and Wong (20). rGM-CSF- and CSF-1-derived macrophages were harvested at day 7 and were recultured at either 1×10^5 or 2×10^5 cells/well in 96-well plates in the absence of CSF. Cultures were treated with medium only or medium plus rIFN-γ for 48 to 72 h. After treatment, monolayers were pulsed with 200 μg/ml of filter-sterilized KLH (Calbiochem-Behring, La Jolla, CA) for 4 h at 37° C. The macrophage cultures were irradiated at 1500 rad (Gammacell 40, ^{137}Cs irradiation unit) at the end of the Ag-pulsing period. After irradiation, the monolayers were washed and co-cultured with 2×10^5 nylon wool-purified lymph node cells (21) isolated from C3H/HeJ mice which had been primed 6 to 7 days previously with 50 μg/foot pad of KLH in CFA (Difco Laboratories, Detroit, MI). The proportion of macrophages to T cells used in this study was based on the approximate range found by others (9, 20) to give strong Ag-specific T cell proliferation by using nylon-wool purified T lymphocytes as the responder population. Macrophages and lymph node cells were co-cultured for 4 days and were then pulsed with [^{3}H] thymidine for 20 h (0.5 μCi/well; NEN, Boston, MA). Cells were harvested onto glass fiber filters and the radioactivity determined by liquid scintillation counting. To analyze the data statistically, an analysis of variance was carried out and the differences between treatment groups compared by using a test for least significant differences (22).

PGE production and assay. After culture of rGM-CSF- and CSF-1-derived macrophage cultures with medium alone, supernatants were harvested at various times and assayed for production of PGE by RIA which had been described elsewhere (23).

RESULTS

Examination of Ia Ag expression on rGM-CSF- and CSF-1-derived macrophages. To compare the number of Ia-positive cells in macrophage cultures derived from bone marrow cells allowed to differentiate in the presence of either rGM-CSF or CSF-1, the macrophages were recultured in the absence of CSF at precise cell densities, as described in *Materials and Methods*. On sequential days over a 3-day culture period the percentage of Ia-positive macrophages was determined by antibody and complement-mediated cytotoxicity (Fig. 1). In untreated macrophages (i.e., those cultured in the presence of medium alone) there was no significant difference in the percent Ia-positive cells in either rGM-CSF- or CSF-1-derived macrophage cultures (approximately 15 to 20%) and this basal level of Ia-positive cells did not change significantly over the 3-day culture period. When cultures of rGM-CSF- and CSF-1-derived macrophages were treated on day 0 with 5.0 U/ml rIFN-γ, both populations of macrophages exhibited an increase in the percentage of Ia-positive cells. This response was most striking after 48 or 72 h of culture (days 2 and 3), with CSF-1-derived macrophages exhibiting the greatest increase in the percent Ia-positive cells. Specifically, at day 3, the percentage of Ia-positive cells increased in CSF-1-derived cultures from approximately 15 to 50% with rIFN-γ, whereas the percentage of Ia-positive cells in the rGM-CSF-derived cultures was increased to only 30%. The viability of both

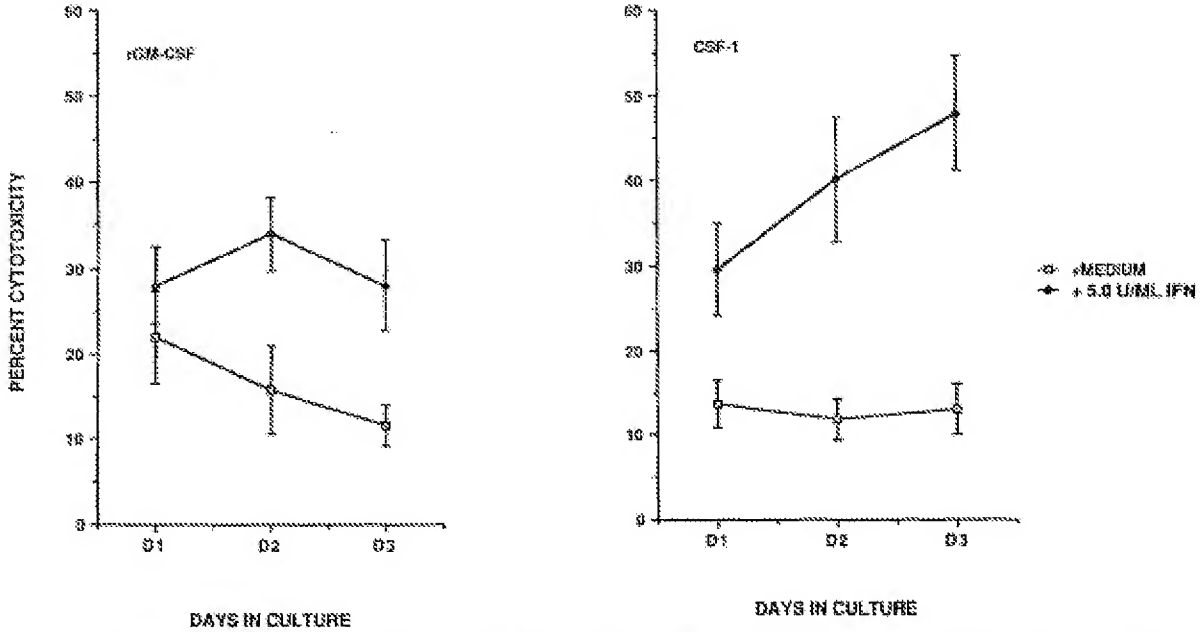

Figure 1. Examination of percent Ia positive cells in rGM-CSF- and CSF-1-derived macrophages. Macrophages (2×10^5) were cultured in the absence of CSF and were assayed on days 1, 2, and 3 after treatment of cultures with either medium or 5.0 U/ml rIFN-γ. The percentage of Ia-positive cells was determined by antibody and complement-mediated cytotoxicity and uptake of trypan blue. Within any individual experiment, 200 cells/culture were scored for viability and duplicate cultures per treatment were assayed. The data represent the arithmetic mean ± SEM for $n = 6$ to 7 individual experiments for rGM-CSF and $n = 10$ to 11 individual experiments for CSF-1 (by using either highly purified or rCSF-1).

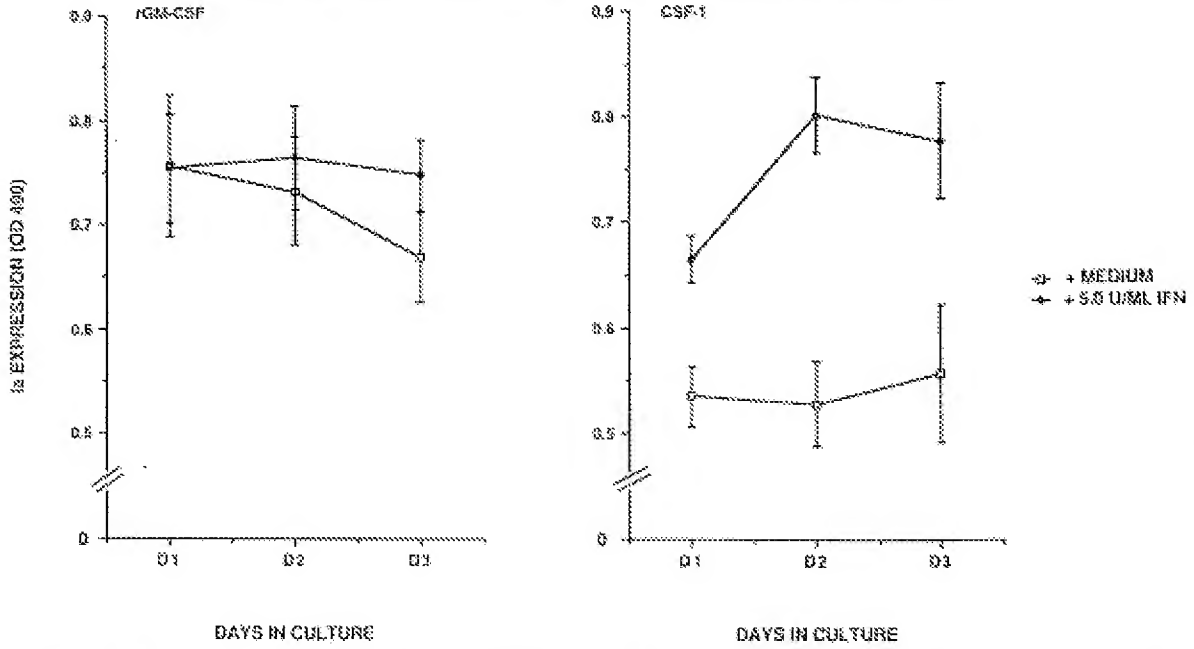

Figure 2. Examination of total Ia expression by ELISA in rGM-CSF- and CSF-1-derived macrophages. Macrophages (3×10^5) were cultured in the absence of CSF and were assayed on days 1, 2, and 3 after treatment with either medium or 5.0 U/ml rIFN-γ. The monolayers were paraformaldehyde-fixed and the total Ia expression determined by ELISA. Within any individual experiment, four wells per treatment were measured. The data represents the arithmetic mean ± SEM for $n = 6$ to 7 individual experiments for rGM-CSF and $n = 10$ to 13 individual experiments for CSF-1 (by using either highly purified or rCSF-1).

cell types remained high (>95%) over the 3-day culture period.

Antibody and complement-mediated cytotoxicity measurements only provide information about the percentage of Ia-positive cells, without regard for the total amount of Ia Ag expressed within the population. ELISA was carried out to compare the total Ia expression within these two populations of cells over a 3-day culture period in the absence and presence of rIFN-γ (Fig. 2). In the absence of rIFN-γ, the basal level of Ia expression (medium alone) remained relatively constant over the 3-day culture period for both CSF-1- and rGM-CSF-derived macrophages. However, rGM-CSF-derived macrophages exhibited a much higher basal level of Ia expression than did CSF-1-derived macrophages. This finding was further substantiated by analysis of steady-state, Ia-specific mRNA in medium-treated rGM-CSF- vs CSF-1-derived cultures. To do this, cytoplasmic RNA was isolated from both populations and was examined by Northern blot and slot blot analysis for quantitation of Ia-specific, steady-state RNA (Fig. 3). By using either method of analysis, it was determined from densitometric scans of autoradiograms that untreated rGM-CSF-derived macrophages possessed approximately fourfold greater quantities of Ia-specific, steady-state mRNA than CSF-1-derived macrophages.

Treatment of rGM-CSF-derived macrophages with rIFN-γ did not increase their level of total Ia expression as measured by ELISA (Fig. 2), i.e., expression of total Ia remained at the already elevated levels observed in medium-treated cultures. However, in the presence of 5.0 U/ml rIFN-γ, CSF-1-derived macrophages increased their total Ia expression to levels exhibited by rGM-CSF-derived cultures.

Determination of Ia density by fluorescent antibody analysis. Since the numbers of Ia-positive cells were approximately equivalent in cultures of medium-treated rGM-CSF- and CSF-1-derived macrophages (Fig. 1), but the total Ia expression, as measured by ELISA, was significantly greater in rGM-CSF-derived cells (Fig. 2), this led to the hypothesis that the density of Ia on rGM-CSF-derived macrophages was significantly greater than on

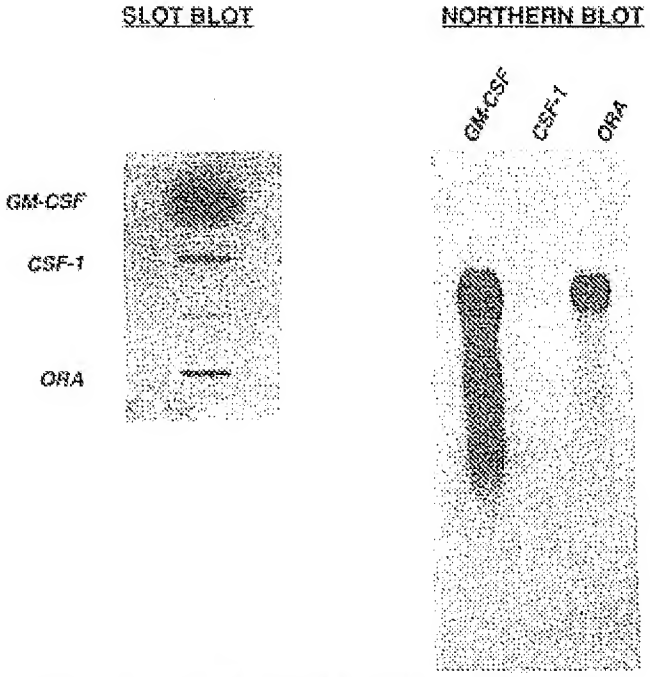

Figure 3. Ia-specific mRNA quantitation in CSF-1- and rGM-CSF-derived macrophages. The autoradiograms from a slot blot (in which 10 μg RNA per slot was applied to the nitrocellulose paper) and a Northern blot (in which 5 μg RNA/lane was applied to the gel) are shown after hybridization to a ^{32}P-cDNA Ia-specific probe. The autoradiogram shown is representative of four separate experiments. The ORA cells were used as a source of constitutively produced, Ia-specific mRNA.

CSF-1-derived cells. To test this hypothesis, the density of Ia on individual macrophages was examined through the use of the ACAS 470 which allows for quantitative analysis of fluorescent cell surface markers on individual cells within an adherent cell population.[5] Through the use of a pseudocolor scale (with white being the highest level of intensity), the degree of fluorescence associated with a particular cell can be visualized. Figure 4 illustrates the distribution of fluorescent staining for Ia expression in medium-treated and rIFN-γ-treated rGM-CSF- and CSF-1-derived macrophages. For each field scanned by the ACAS 470, comparability of cell density per field was verified by phase contrast microscopy. Figure 4, *A* and *B*, demonstrates that although the number of Ia-positive cells were found to be equivalent (Fig. 1), the density of Ia expression on medium-treated, CSF-1-derived macrophages (Fig. 4*A*) was so low, when compared with the fluorescence exhibited by rGM-CSF-derived macrophages (Fig. 4*B*), that most of the CSF-1-derived cells could not be visualized. However, after rIFN-γ treatment (Fig. 4*C*), there was an increase in the number of fluorescent cells within the CSF-1-derived population, as well as a marked increase in the intensity of fluorescence. In addition, the density of Ia per cell was still less than that seen in medium- or rIFN-γ-treated rGM-CSF-derived cells. Treatment of rGM-CSF-derived cultures with rIFN-γ failed to alter significantly the number of positive cells detected by the ACAS 470 or the already high density of Ia per cell (Fig. 4*D*).

Measurement of Ag-induced T cell proliferation by rGM-CSF- and CSF-1-derived macrophages. The relationship of Ia Ag expression to the ability of macrophages to function as Ag-presenting cells has been well-documented (6, 9, 20, 24). Since differences in Ia expression were observed in these two cell populations, the capacities of these cultures to induce Ag-specific T cell proliferation was also assessed. In Figure 5, rGM-CSF- or CSF-

[5] The potential damage to adherent cells which must be mechanically or enzymatically detached for analysis by standard flow cytometry has always presented a technical problem for fluorescent analysis of cell-surface markers. Therefore, this method provides more flexibility because cells treated in culture can be examined without having to be detached for analysis. Although the ACAS 470 provides a graded pseudocolor intensity scale from blue to white, the black and white photography of the scanned color image provides adequate visualization of density differences.

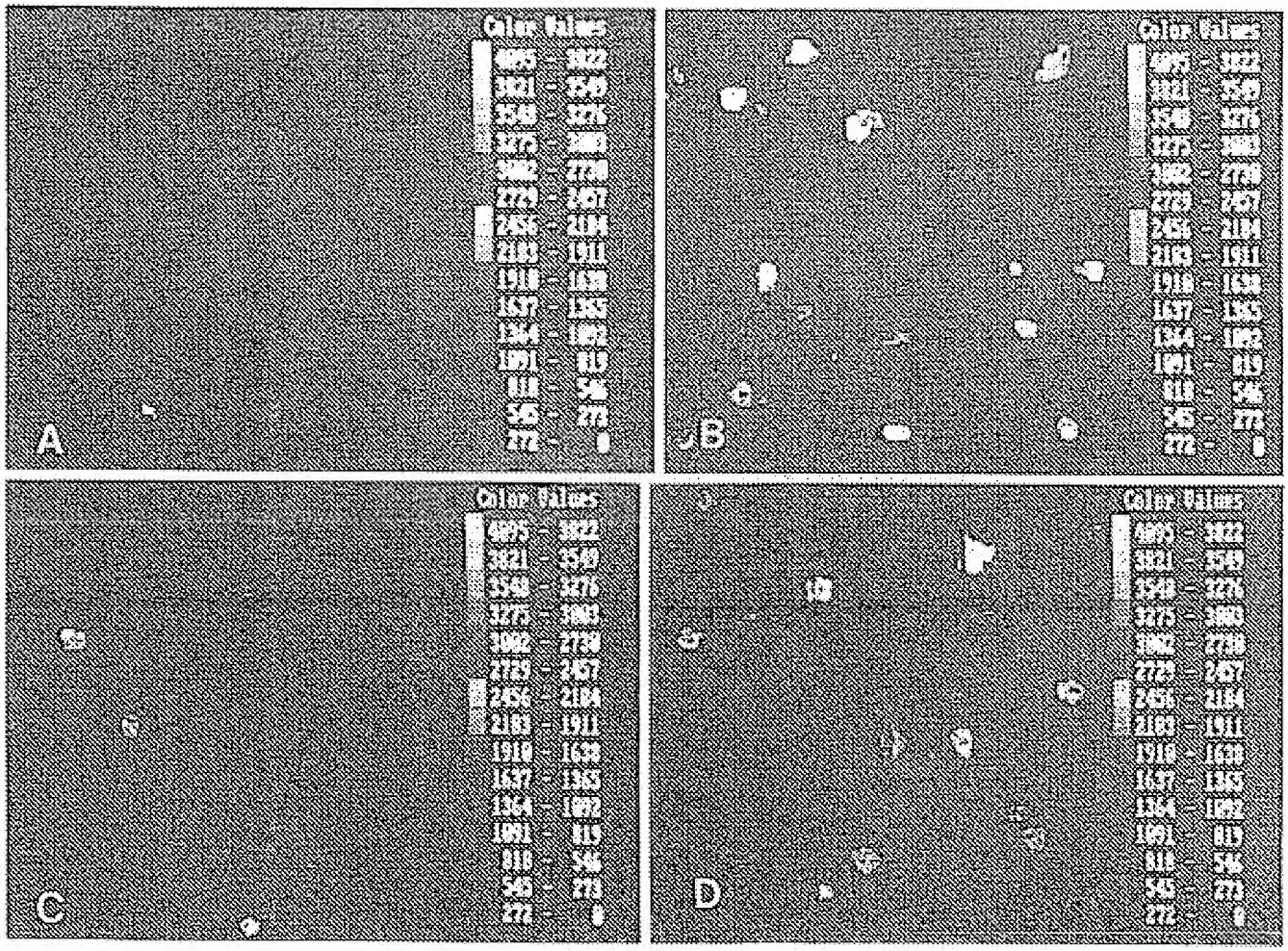

Figure 4. Ia density analysis by fluorescence in rGM-CSF- and CSF-1-derived marrophages. Macrophages (1×10^5/culture) were treated with medium or 5.0 U/ml rIFN-γ for 2 to 3 days and were analyzed for Ia Ag expression using the ACAS 470. To control for background fluorescence, cells treated with fluorescenated secondary antibody only were included and showed little detectable fluorescence. The fields scanned on the control cultures were established as the threshold and were subtracted from all of the other scans so to eliminate this low background of nonspecific fluorescence. *A*, Medium-treated CSF-1-derived macrophages; *B*, medium-treated rGM-CSF-derived macrophages; *C*, rIFN-γ-treated CSF-1-derived macrophages; *D*, rIFN-γ-treated, rGM-CSF-derived macrophages. Each figure represents a single representative field which was verified by phase contrast microscopy to contain approximately the same number of cells (i.e., 80 to 110 cells/field).

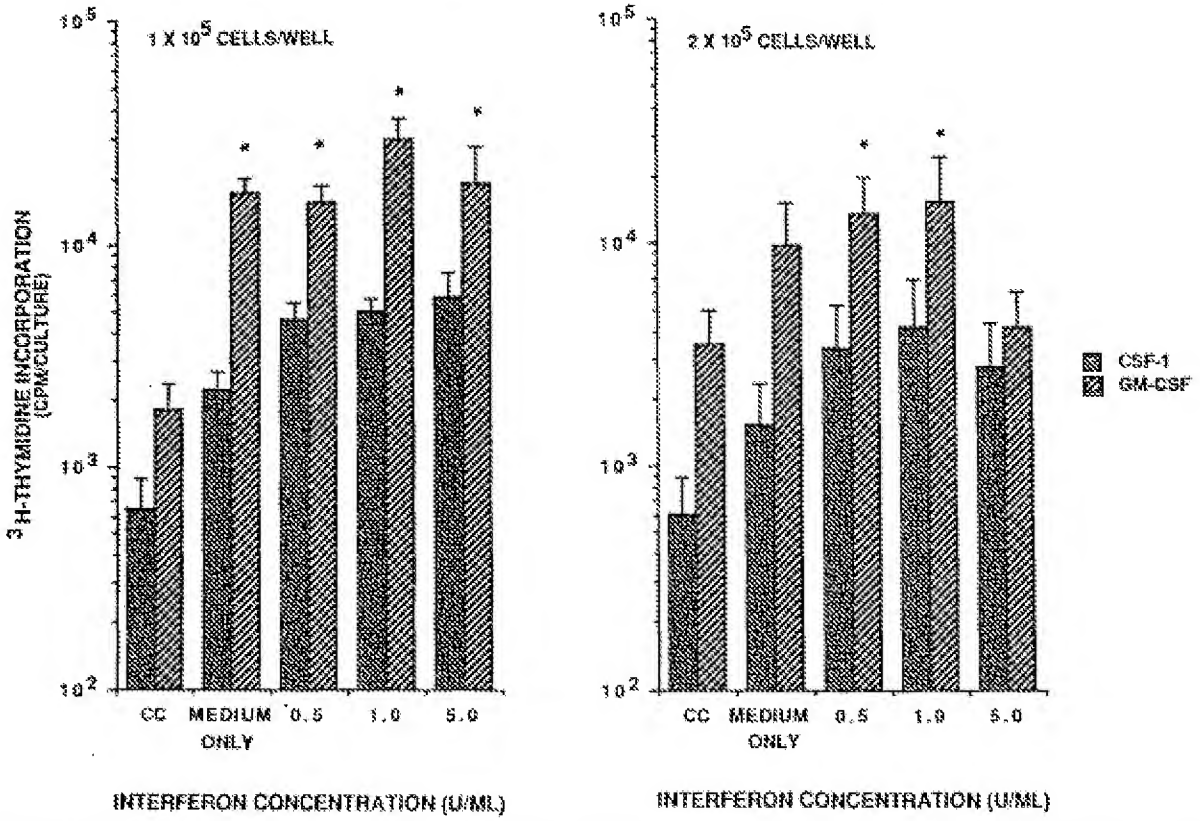

Figure 5. Comparison of Ag-specific T cell proliferation induction by rGM-CSF- and CSF-1-derived macrophages. Macrophages were cultured at 1×10^5/culture (A) or 2×10^5/culture (B) in the absence or presence of rIFN-γ for 2 to 3 days. At that time they were pulsed with Ag (KLH) for 4 h, irradiated, and co-cultured with KLH-primed, nylon wool-purified, lymph node cells T cells for an additional 4 d. Tritiated thymidine was added to cultures on day 4 and the radioactive incorporation measured by liquid scintillation counting. The data represent the arithmetic mean ± SEM for $n = 4$ individual experiments. The cell control (CC) values (cpm incorporated into macrophages and T cells in the absence of Ag) have not been subtracted from the experiment values, but rather, are included as part of the graph. Within certain treatment groups (those indicated by an asterisk), rGM-CSF-derived macrophages induced significantly greater Ag-specific T cell proliferation ($p \leq 0.05$) than CSF-1-derived macrophages.

1-derived macrophages were cultured at two different cell concentrations (i.e., 1×10^5 and 2×10^5 cells/culture) and were incubated with medium or rIFN-γ (0.5, 1.0, or 5.0 U/ml) for 2 or 3 days. At that time, cells were Ag-pulsed with KLH and irradiated. The ability of T cells derived from KLH-primed mice to proliferate in response to Ag when presented by these macrophages was measured by uptake of tritiated thymidine. In the cell controls (macrophages plus T cells in the absence of Ag), rGM-CSF-derived macrophages were more stimulatory for T cells than CSF-1-derived macrophages. Other controls included T cells alone or macrophages alone and consistently incorporated less than 500 cpm/culture (data not shown). When incubated with medium alone (before Ag pulsing), rGM-CSF-derived macrophages were capable of stimulating greater T cell proliferation than medium-treated CSF-1-derived cells. rIFN-γ treatment had little effect on the ability of rGM-CSF-derived macrophages to stimulate T cell proliferation; however, treatment of CSF-1-derived macrophages increased their ability to stimulate Ag-specific T cell proliferation slightly, although this difference was not found to be statistically significant. At 2×10^5 macrophages/well, rGM-CSF-derived macrophages continued to exhibit greater Ag-specific T cell proliferation than CSF-1-derived macrophages, but at the highest concentration of rIFN-γ used (5.0 U/ml), a depression of Ag-specific T cell proliferation induced by the rGM-CSF-derived macrophages was observed.

Basal expression of PGE production by rGM-CSF- and CSF-1-derived macrophages. PG have been shown by a number of investigators to inhibit Ia expression in both resident and elicited peritoneal macrophages (25, 26). PG have also been shown to inhibit mitogen-induced T lymphocyte proliferation (27–30). Since CSF-1-derived macrophages exhibit lower basal levels of Ia expression than rGM-CSF-derived macrophages (Figs. 2 and 4), as well as a lesser ability to induce proliferation of T lymphocytes in the absence or presence of Ag (Fig. 5), we examined the level of PGE produced in rGM-CSF- vs CSF-1-derived cultures (Table I). Macrophages derived from bone marrow progenitors in the presence of rGM-CSF produced approximately 10-fold higher levels of PGE than macrophages derived in the presence of CSF-1.

DISCUSSION

It has been recognized for many years that Ia Ag are essential for the appropriate presentation of Ag by APC

TABLE I

PGE production by GM-CSF- and CSF-1-derived macrophages[a]

Macrophage Type	PGE Production (pg/ml)[b]
rGM-CSF-derived	11.5 [3.7–19.3]
CSF-1-derived	1.5 [0.3–2.7]

[a] Supernatants from medium-treated cultures were harvested at either 24 or 48 h and were assayed for PGE activity by RIA.

[b] The data represent the arithmetic mean derived from eight to nine separate experiments and the 95% confidence limits (in brackets) for each treatment group.

to specific T cells (6–9). More recently attention has focused on the mechanisms by which Ia Ag expression is regulated. Specifically, IFN-γ has been shown to be a principal lymphokine responsible for up-regulating Ia Ag expression on macrophages (5, 10–12, 13), as well as on other cell types (32–36), and in some studies, enhanced expression of Ia has been correlated with increased Ia-dependent accessory functions (9, 10, 31, 37). These studies were carried out primarily on macrophage populations which express low levels of Ia in the absence of inducer. However, it has also been recognized for some time that subpopulations of highly Ia-positive macrophages exist (13, 37), even in athymic nude mice (14), where the availability of endogenously produced, T cell-derived IFN-γ to induce and/or maintain Ia expression would be markedly limited. This raises the possibility that other mechanisms may contribute to the development of certain populations of Ia-positive macrophages within the body.

GM-CSF and CSF-1 are principally responsible for the differentiation of macrophages along the monocytic lineage (1). At this time, it is not clear whether these two cytokines act on discrete or overlapping populations of bone marrow progenitors (38, 39) or whether the mature macrophages which arise under the influence of these distinct factors are functionally distinct populations (16, 39–41). Early studies by Lee and Wong (20, 42) demonstrated that macrophages derived from bone marrow cells under the influence of crude preparations of fibroblast-derived CSF (i.e., shown by others to be principally CSF-1 (43) or a CSF found in endotoxin-stimulated lung-conditioned medium (i.e., either a GM-CSF or a mixture of CSF) (44, 45) differed in their expression of Ia. Specifically those cells grown in the crude CSF-1 preparation exhibited lower Ia Ag expression and "Ag-presenting" activity than cells grown in lung-conditioned medium, but the expression of Ia on the CSF-1-derived cells could be augmented by exposure of the cells to crude lymphokine preparations. Similarly, Calamai et al. (46) showed that bone marrow cells cultured in crude fibroblast supernatants gave rise to macrophages which expressed low levels of Ia, but could be induced to become Ia-positive after exposure to lymphokines. These authors raised the possibility that CSF may act as an antagonist to the stimulus for Ia induction.

By using recombinant and highly purified preparations of GM-CSF and CSF-1, we have confirmed and extended these findings. Specifically, macrophages derived under the influence of recombinant or purified, natural CSF-1 possess lower basal levels of Ia Ag expression, express reduced levels of Ia-specific mRNA, and are significantly less capable of inducing Ag-specific T cell proliferation when compared with rGM-CSF-derived macrophages. Thus, rGM-CSF gives rise to a population of mature macrophages which is intrinsically more Ia-positive and more capable of Ia-related functions than CSF-1-derived macrophages. Inasmuch as PG have been shown to be antiproliferative to lymphocytes (27–30) and because PG have been shown to down-regulate Ia Ag expression on macrophages (25, 26), the potential role of PGE in the differences observed in basal Ia expression and function of these two populations was examined. The finding that rGM-CSF-derived macrophages produce approximately 10-fold greater levels of PGE than CSF-1-derived macrophages would argue against the possible role of PGE in the maintenance of low basal Ia expression in CSF-1-derived macrophages. In addition, our findings are in agreement with those of Vespa et al. (47) in which PGE had no effect on the modulation of Ia expression in a constitutive Ia-expressing cell line, while demonstrating its inhibitory effect on transient Ia expression in macrophages after induction by lymphokines.

Another possible explanation for the differences in basal Ia expression exhibited by these two populations may exist in the observation that Ia expression has been shown previously to be down-regulated by IFN-α/β (48, 49). It has been demonstrated that CSF-1-derived macrophages produce detectable levels of IFN-α/β and exhibit many IFN-α/β-dependent functions (i.e., resistance to virus infection which is reversible by treatment of cultures with antibody to IFN-α/β and increased FcR expression (12, 50–52). This is in striking contrast to the failure of rGM-CSF-derived macrophages to resist virus infection, the ability to protect these cells by pretreatment with IFN-α/β, as well as their relatively low FcR capacity (16, 50). Thus, it is possible that endogenous IFN-α/β production plays a significant role in the maintenance of low basal Ia expression in CSF-1-derived macrophages as well as in the overexpression of Ia in rGM-CSF-derived macrophages.

Although rIFN-γ was found to increase Ia expression on CSF-1-derived macrophages, the ability of IFN-γ to increase the capacity of these cells to drive Ag-specific T cell proliferation was only minimal (Fig. 5). The ability of macrophages to function as "APC" is a complex process which involves Ag uptake, Ag processing, presentation of Ag in the context of appropriate Ia Ag, as well as the presence of additional macrophage-derived signals (reviewed in Ref. 24). The inability of increased total Ia expression in CSF-1-derived macrophages to result in a functional increase in Ag-specific T cell stimulation poses an interesting question concerning the relevance of total Ia expression. One possible explanation for this finding may be that the density of Ia Ag available to the Ag-primed T cells is critical for maximal stimulation and may suggest that a threshold level of Ia is required for Ag-specific T cell proliferation. It is clear from the fluorescent antibody analysis (Fig. 4C) that the density of Ia Ag on rIFN-γ-treated, CSF-1-derived macrophages still remains less than that seen in rGM-CSF-derived cells (medium- or rIFN-γ-treated), even though the total Ia (as measured by ELISA) is equivalent and the number of Ia-positive macrophages is actually greater (as measured by direct cytotoxicity). Thus, the density of Ia on individual CSF-1-derived macrophages may simply be inadequate (i.e., below threshold levels) for optimal Ag presentation. Alternatively, reduced presentation function may reflect a failure of CSF-1-derived macrophages to process Ag correctly or to develop some additional cell surface marker(s) which is (are) necessary for T cell stimulation, such as IL-1 (55–57). Although we have no data which compare the processing capabilities of these two population of cells, the possible involvement of IL-1 in the differences observed with respect to T cell-induced proliferation has been examined. No detectable IL-1 was found in supernatants of medium-treated rGM-CSF- or CSF-1-derived macrophages (data not shown). However, the role of membrane-bound IL-1 may be of importance

and is currently under investigation. Last, the finding that exposure of rGM-CSF-derived macrophages to high levels of rIFN-γ led to an inhibition of Ag-specific T cell proliferation at high cell concentration is consistent with previous reports of IFN-γ-induced suppression of Ag-stimulated lymphocyte proliferation (58, 59).

In conclusion, this report establishes a functional distinction between macrophages derived under the influence of GM-CSF vs CSF-1. Since the production of GM-CSF has been ascribed to cell types other than T cells (60–62), it is possible that this form of CSF is responsible for the development of Ia-positive macrophages in specific organs in the absence of a sustained IFN-γ signal.

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EXHIBIT 3

REGULATION OF MHC CLASS II GENE EXPRESSION IN MACROPHAGES BY HEMATOPOIETIC COLONY-STIMULATING FACTORS (CSF)

Induction by Granulocyte/Macrophage CSF and Inhibition by CSF-1

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The class II MHC molecules expressed on antigen-presenting cells are highly polymorphic, heterodimeric cell surface glycoproteins that bind and present proteolytic fragments of foreign antigens to T cells to initiate the immune response (1). MHC class II (Ia) molecules are composed of a 33–36-kD α subunit noncovalently associated with a 24–28-kD β subunit. A third nonpolymorphic invariant chain (Ii) of 31 kD may be found associated with Ia molecules in the cytoplasm and on the cell surface. In contrast to the constitutive expression of Ia in B cells, the expression of Ia and Ii in different macrophage populations has been reported to be induced by IFN-γ (2, 3), IL-4 (4), and granulocyte/macrophage CSF (GM-CSF)[1] (5, 6). Using highly purified macrophages derived from in vitro bone marrow cultures, we have determined that the recombinant hematopoietic colony-stimulating factors CSF-1 and GM-CSF differently regulate Ia and Ii gene and protein expression in macrophages.

Materials and Methods

Bone Marrow Cultures. Bone marrow macrophages (BMM) were derived from short term in vitro cultures of DBA/2 or C3H/HeJ murine bone marrow cells cultured at 2×10^5 nucleated cells/ml in α-MEM medium supplemented with L929 cell–conditioned medium containing CSF-1 (LCM) and 10% FCS (HyClone Laboratories, Logan, UT) as previously described (7). After 6 d, the adherent macrophages in these cultures were washed in PBS and were then stimulated with fresh α-MEM supplemented with either recombinant CSF-1 (>95% pure by SDS-PAGE; used in our experiments at 1,000 CFU/ml; Cetus Corp., Emeryville, CA), recombinant murine GM-CSF (obtained either from Immunex, Seattle, WA [>95% pure; 10^8 CFU/mg protein] or the kind gift of J. Gasson, University of California, Los Angeles [derived from COS cell supernatants; >95% pure] used at 500–1,000

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[1] *Abbreviations used in this paper:* BMM, bone marrow-derived macrophages; CSF-1, monocyte CSF; GM-CSF, granulocyte/macrophage CSF; LCM, L-929 cell-conditioned medium.

CFU/ml in our experiments); recombinant murine IFN-γ (1.3×10^7 U/mg protein; used at 20 U/ml; Genentech, San Francisco, CA), IFN-α/β (250 U/ml; Lee Biomolecular, San Diego, CA), or IL-3 (1,000 U/ml; Genzyme, Boston, MA). All media and supplements contained <0.1 ng/ml of endotoxin as determined by the limulus amoebocyte lysate assay.

RNA Isolation and Hybridization Assays. Total cellular RNA was isolated, electrophoresed, transferred to nitrocellulose, and hybridized as previously described (17). DNA probes used in this study included invariant chain (Ii), 1.17-kb Pst I fragment (8); Ia-A_β^e, 1.7-kb Hind III fragment (9); and pHH2a, an MHC class I (H-$2K^k$) probe/2.0-kb Hba I-Sac I fragment (10).

Flow Cytometric Analysis of Class II Antigen Expression. Viable BMM were centrifuged and resuspended in PBS supplemented with 0.1% sodium azide and 0.5% BSA (PAB) at a concentration of 2×10^7 cells/ml and kept on ice. Membrane expression of Ia was determined using a rat anti-mouse Ia with specificity for $IA^{b/s}$ and $IE^{d/k}$ (IgG2b, TIB120; American Type Culture Collection, Rockville, MD). Because of this broad specificity, a haplotype control was not used in these experiments. To prevent nonspecific Fc binding of the rat anti-Ia, 10^6 BMM (in 50 μl) were first preincubated with 10 μg (in 10 μl) of purified mouse normal Ig (Sigma Chemical Co., St. Louis, MO) for 10 min. We have previously determined that this preincubation inhibits the binding of directly fluoresceinated mouse IgG2a myeloma protein to BMM (data not shown). Without washing, 1 μg (in 10 μl) of the rat anti-Ia was added to the cells and the cells were incubated for 15 min. After washing in 4 ml PAB, the cells were incubated for 15 min with 1 μg (in 10 μl) of fluoresceinated goat anti-rat Ig (with no crossreactivity for mouse Ig; Caltag, San Francisco, CA). All antibody reagents used were >90% Ig protein. After a final wash, the cells were resuspended in 1 ml PAB containing 2 μg of propidium iodide (to exclude dead cells from analysis) and were analyzed by flow cytometry. A single argon laser operating at 488 nM was used to measure low angle light scatter and to excite fluorescein and propidium iodide. Emission of fluorescein was detected at 530 ± 15 nM, while propidium iodide emission was detected above 590 nM.

Cell Cycle Analysis. To measure the fraction of proliferating BMM in S phase, 30 μM bromodeoxyuridine (BrdU; Sigma Chemical Co.) was added to the culture medium 30 minutes before cell harvest. Cells were collected, centrifuged, resuspended in 0.5 ml saline, and fixed in 5 ml of 70% ethanol in water for 24 h. Fixed cells were centrifuged and the pellet was resuspended in 5 ml of 4 N HCl with 0.5% Tween 20. After vortexing, cells were incubated 30 min at 37°C; washed with 2.5 ml of 0.1 M sodium borate, pH 8.5; and the pellet was resuspended in 30 μl PBS with 0.5% Tween 20. Cells were then stained with fluoresceinated anti-BrdU (Becton Dickinson & Co., Mountain View, CA) for 30 min at room temperature, washed, counterstained with 20 μg/ml propidium iodide containing 50 μg/ml RNase, and analyzed by flow cytometry using the same parameters described above.

Results

Suppression of Ia and Ii Gene and Protein Expression by CSF-1. In studies designed to examine CSF-1 inducible genes in monocytic cells, we noted that BMM expressed variable basal levels of Ia mRNA. Adherent BMM, derived directly from day 6 bone marrow cultures supplemented with LCM (see Materials and Methods), contained essentially undetectable levels of Ia-Aβ and Ii mRNA (Fig. 1 *A*), as well as undetectable levels of Ia-Aα mRNA (data not shown). When these day 6 adherent BMM were washed and refed with fresh medium containing serum only, Ia and Ii mRNA transcripts remained at essentially undetectable levels until after 24 h of culture, at which time there was an abrupt 20-fold increase in the basal levels of both Ia and Ii mRNA (Fig. 1 *A*). Ia and Ii mRNA could still be detected at 48 h (Fig. 1 *A*). BMM viability, assessed by dye exclusion, was maintained under these experimental conditions (data not shown). In contrast to Ia and Ii, the expression of MHC class I mRNA (H-$2K^k$) was unaltered during this experiment (Fig. 1 *A*).

To determine the effects of CSF-1 on basal levels of Ia expression in BMM, recom-

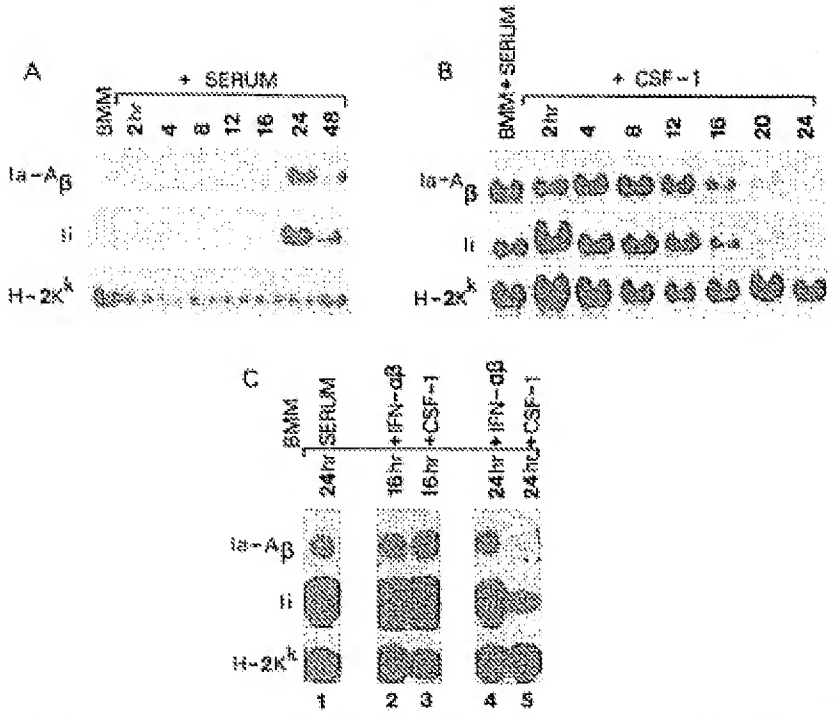

FIGURE 1. (*A*) Total RNA was isolated from BMM derived directly from day 6 in vitro cultures supplemented with LCM (BMM, lane *1*) or from day 6 BMM that were washed, refed with medium containing serum alone, and then cultured for 2–48 h (lanes *2–6*). 10 μg of total RNA from each time point was hybridized in Northern analysis with probes for MHC class II Ia-$A\beta^k$, Ii, and MHC class I H-$2K^k$. (*B*) Day 6 BMM were cultured in serum alone for 24 h to allow maximal basal expression of Ia and Ii (BMM + serum, lane *1*). These cells were then washed, refed with medium containing recombinant CSF-1, and cultured for 2–24 h (lanes *2–6*). 10 μg of total RNA from each time point was hybridized in Northern analysis with probes described in (*A*). (*C*) Total RNA was isolated from day 6 adherent BMM which had been cultured for 24 h in serum alone to allow maximal basal Ia expression (lane *1*). These cells were then washed and recultured for 16 (lane *2*) or 24 h (lane *4*) in medium containing IFN-α/β; or alternatively, in medium containing recombinant CSF-1 for 16 (lane *3*) or 24 h (lane 5). 10 μg of total RNA from each experimental variable was analyzed in Northern analysis with probes described in (*A*).

binant CSF-1 was added back to the cells that had been cultured in serum alone for 24 h and that now expressed maximal levels of Ia and Ii mRNA. Ia and Ii transcript levels were unaltered until after 16 h of culture with CSF-1, at which time there was a 25% decrease in mRNA expression (Fig. 1 *B*). By 24 h of culture with CSF-1, Ia and Ii transcripts were essentially undetectable (Fig. 1 *B*). Expression of H-$2K^k$ mRNA was essentially unaffected.

Since the suppression of basal levels of Ia by recombinant CSF-1 occurred over a 24-h time course (Fig. 1 *B*), we wanted to determine whether this suppression might be mediated by the autocrine effects of IFN-α/β, or prostaglandin E_2 (PGE_2) induced in the BMM by CSF-1 (11–13). To test this possibility, day 6 BMM were first cultured for 24 h in fresh medium containing serum alone to allow maximal expres-

sion of basal levels of Ia and Ii. Fresh medium containing serum and either recombinant CSF-1, IFN-α/β, or PGE_2 was then added directly to the BMM. After 16 (Fig. 1 *C*, lane *2*) or 24 (Fig. 1 *C*, lane *4*) h of culture with IFN-α/β, no suppression of the basal levels of Ia or Ii mRNA was seen. A similar result was obtained when cultures were supplemented with PGE_2 (data not shown). In contrast, basal levels of Ia and Ii mRNA were inhibited by CSF-1, but not until after 24 h of culture (Fig. 1 *C*, lane *5*), as previously shown (Fig. 1 *B*). The suppression of Ia and Ii gene expression by CSF-1 was not due to contaminating endotoxin (LPS) since medium and supplements contained <0.1 ng/ml endotoxin and similar experiments performed in LPS-hyporesponsive C3H/HeJ mice produced identical results (data not shown).

Correlating with these changes in Ia and Ii gene expression, very low levels of Ia glycoproteins could be detected on day 6 BMM that had been cultured in serum alone for an additional 24 h in the absence of CSF-1 (Fig. 2 *B*), while no expression of Ia could be detected on the cell surface of BMM derived directly from the day 6 cultures (data not shown) or on day 6 BMM which had been cultured with recombinant CSF-1 for an additional 24 h (Fig. 2 *A*).

Induction of Ia and Ii Gene Expression by GM-CSF. In contrast to the inhibitory effects of CSF-1, recombinant murine GM-CSF induced maximal levels of Ia-Aβ and Ii mRNA after 24 h of culture (Fig. 3 *A*, lane *3*), although increased transcript levels could be detected as early as 8 h (data not shown). The levels of Ia and Ii mRNA induced by GM-CSF (1,000 U/ml) were similar to the levels induced with IFN-γ (20 U/ml) (Fig. 3 *A*, lane *4*). No induction of Ia was seen with doses of GM-CSF <150 U/ml (data not shown) and no synergistic effect was noted with the simultaneous addition of GM-CSF and IFN-γ (data not shown). In contrast, IL-3 had no effect on Ia and Ii expression (data not shown). Additionally, no significant modulation of H-$2K^k$ mRNA expression was noted (Fig. 3 *A*).

Paralleling the increases in Ia mRNA, both GM-CSF and IFN-γ significantly increased the expression of Ia proteins on the BMM cell surface (Fig. 2, *C* and *D*). Levels of Ia gene and protein expression *induced* by GM-CSF and IFN-γ in the BMM were ~10–20-fold higher than the maximal *basal* levels of expression (Fig. 3 *A*).

Suppression of the IFN-γ and GM-CSF Induction of Ia by CSF-1. To determine if recombinant CSF-1 could suppress the induction of Ia by GM-CSF and IFN-γ, as it had suppressed the basal levels of Ia expression, these factors were added simultaneously to BMM. Day 6 adherent BMM were washed and refed with fresh medium containing either GM-CSF, the combination of CSF-1 + GM-CSF, IFN-γ, or the combination of CSF-1 + IFN-γ, and cultured for 24 h. As before, both GM-CSF (Fig. 3 *B*, lane *1*) and IFN-γ (Fig. 3 *B*, lane *3*) induced high levels of Ia and Ii mRNA. However, when CSF-1 was also present, the Ia induction was markedly suppressed (Fig. 3 *B*, lanes *2* and *4*). In multiple experiments, CSF-1 markedly inhibited the induction of Ia by GM-CSF, while the IFN-γ–mediated induction of Ia mRNA was suppressed by 50–90% and Ii mRNA levels were suppressed to a somewhat lesser degree (25–50%). CSF-1 (1,000 U/ml) was capable of suppressing the IFN-γ induction of Ia and Ii over a dose range of 10–100 U/ml of IFN-γ (data not shown). Lower doses of CSF-1 were not tested.

The CSF-1-mediated suppression of the IFN-γ and GM-CSF induction of Ia mRNA was paralleled by decreased expression of Ia glycoproteins on the BMM cell surface. Compared with the induced levels of Ia detected on BMM treated with IFN-γ alone

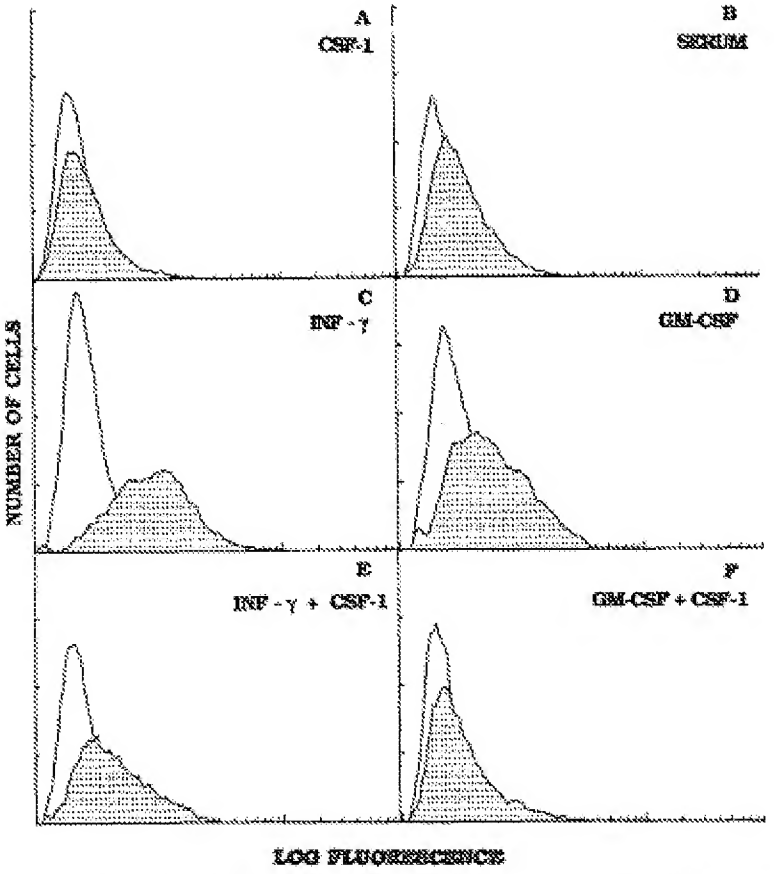

FIGURE 2. Day 6 adherent BMM were washed and recultured for an additional 24 h in medium containing recombinant CSF-1 (*A*), serum alone (*B*), IFN-γ (*C*), recombinant GM-CSF (*D*), the combination of IFN-γ + CSF-1 (*E*), or the combination of GM-CSF + CSF-1 (*F*). The cells were then harvested, stained with the anti-Ia reagents and analyzed by flow cytometry as described in Materials and Methods. The shift in log fluorescence due to specific binding of anti-Ia is displayed on the histograms as a filled histogram relative to the background control (fluorescence of the BMM stained with the fluoresceinated second step antibody alone) displayed as an unfilled histogram.

(Fig. 2 *C*), Ia levels in BMM cultured simultaneously with IFN-γ and CSF-1 were reduced by more than 50% (Fig. 2 *E*). Similarly, Ia expression in BMM treated with the combination of CSF-1 + GM-CSF was also suppressed (Fig. 2 *F*), when compared with Ia levels induced by GM-CSF (Fig. 2 *D*).

To determine if suppression or induction of cytoplasmic steady-state levels of Ia and Ii mRNA by CSF-1, GM-CSF, and IFN-γ were due to the production of new transcripts in the nucleus, or due to posttranscriptional mechanisms, in vitro nuclear transcriptional assays were performed. In triplicate experiments, no significant differences were noted in the level of transcription of Ia-Aβ when day 6 BMM were washed and recultured for 24 h in fresh medium containing serum alone, CSF-1,

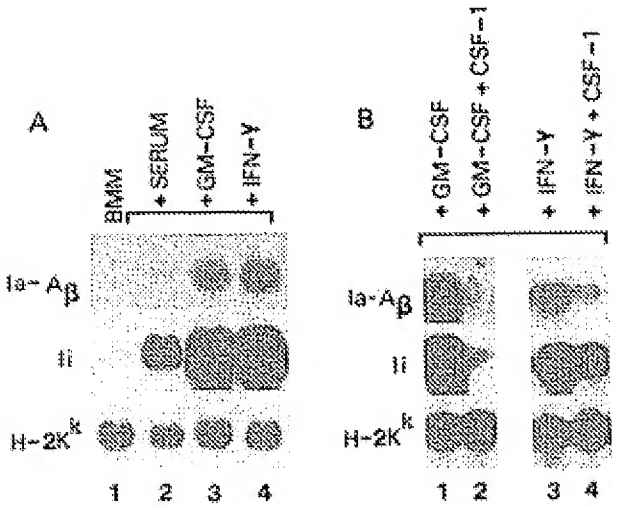

FIGURE 3. (*A*) Total RNA was isolated directly from day 6 adherent BMM (lane *1*) or from BMM that had been washed and refed with medium containing either serum alone (*lane 2*), GM-CSF (lane *3*), or IFN-γ (lane *4*) and cultured for an additional 24 h. 10 μg of total RNA from each variable was hybridized in Northern analysis with probes for Ia, Ii, and H-2K^k. (*B*) 10 μg of total RNA was isolated from day 6 BMM cultured for an additional 24 h in GM-CSF (lane *1*), GM-CSF + CSF-1 (lane *2*), IFN-γ (lane *3*), or IFN-γ + CSF-1 (lane *4*) and was hybridized in Northern analysis with the probes described in *A*.

or GM-CSF (data not shown). In response to IFN-γ, the BMM displayed only a 1-1.7-fold increase in the level of transcription of class Ia-Aβ after 24 h of culture, (data not shown), similar to recent reports (14). No significant changes in the level Ia transcription were observed when BMM were cultured with these factors for shorter time periods of 30 min and 8 h (data not shown).

Relationship of the Suppression and Induction of Ia to the Proliferative Status of BMM. Previous studies by Calamai et al. (15) suggested that the induction of macrophage proliferation by LCM led to a cell cycle–dependent inhibition of Ia expression. To determine if there was a relationship between the proliferative status of the BMM and Ia expression, we determined the S phase fraction of BMM at each time point in which Ia expression had been assessed. Day 6 adherent BMM, derived directly from cultures supplemented with LCM, had 32% of cells in S phase and had no evidence of Ia gene or protein expression (Figs. 1 *A* and 2 *A*). Similarly, BMM cultured for an additional 24 h with recombinant CSF-1 had 29% of cells in S phase and had essentially undetectable Ia expression (Figs. 1, *B* and *C*; 2 *A*).

In contrast, day 6 BMM cultured for an additional 24 h in serum alone in the absence of CSF-1, or day 6 BMM cultured for 24 h with GM-CSF or IFN-γ alone were not in cell cycle (with <2%, <1%, and <4% of cells in S phase, respectively); and, the nonproliferating BMM derived from each of these conditions expressed both Ia and Ii mRNA (Fig. 3) and proteins (Fig. 2). Culture of the BMM for 24 h with CSF-1 + IFN-γ or CSF-1 + GM-CSF maintained the proliferative state of the macrophages (with 30% and 22% of the cells in S phase, respectively). These conditions were associated with suppression of the IFN-γ and GM-CSF-mediated induction of Ia expression (Figs. 2, *E* and *F*; 3 *B*). Thus, in every case in which CSF-1 was present and proliferation was induced, Ia expression was markedly inhibited.

Discussion

In our studies, hematopoietic CSF were shown to modulate the levels of Ia expression in BMM. We determined that BMM have low basal levels of Ia and Ii gene

and protein expression. While IL-3 had no effect, both GM-CSF and IFN-γ increased the basal levels of Ia expression 10–20-fold. In contrast, recombinant CSF-1 suppressed both the basal levels of Ia expression and inhibited the induction Ia by GM-CSF and IFN-γ. Our preliminary studies investigating the molecular mechanisms that regulate Ia gene expression in BMM suggested that both transcriptional and posttranscriptional mechanisms may be operative. Only a 1–1.7-fold increase in transcription of Ia-Aβ^d was noted in response to IFN-γ, at a time in which steady-state levels of Ia-Aβ^d mRNA transcripts in the cytoplasm increased >20–30-fold. Whether these changes in the level of transcription can account solely for the large increase in cytoplasmic mRNA (without involving additional posttranscriptional mechanisms) remains to be determined.

The delay in the CSF-1–mediated suppression of basal levels of Ia suggested an indirect mechanism. We determined that suppression was not due to the autocrine production of either IFN-α/β or PGE_2. Although IFN-α/β was previously shown to inhibit the IFN-γ–mediated induction of Ia (13), no previous data has been reported on the effects of IFN-α/β on basal levels of Ia in macrophages. In agreement with our experiments, recent studies have also reported that PGE_2 has no effect on the basal levels of Ia expression in macrophages (6), even though PGE_2 may inhibit the induction of Ia by IFN-γ (12). Our work has determined that the inhibition of Ia expression by CSF-1 is correlated with the induction of cellular proliferation, extending the earlier work of Calamai et al. (15). To insure that the adherent BMM were still in cell cycle on day 6, initial bone marrow cultures were plated at extremely low densities; when plated at high initial densities, day 6 BMM may be confluent and quiescent, due to consumption of all of the CSF-1 in the culture medium (7). When day 6 BMM derived from the low density cultures are rendered quiescent by removal of LCM and are then stimulated to proliferate by the readdition of recombinant CSF-1, the cells enter the S phase of the cell cycle by 20–24 h (7). This time frame of S phase entry correlates precisely with the time of inhibition of Ia expression by CSF-1 (Fig. 1 *B*). Whether the suppression of Ia is due directly or indirectly to CSF-1 or is a consequence of the induction of cellular proliferation remains to be determined.

Even though CSF-1 may inhibit Ia expression through cell cycle–dependent mechanisms, Ia expression can certainly be inhibited in macrophages by other cell cycle–independent mechanisms, such as those initiated by LPS (16) and TNF-α (Adams, D., personal communication). TNF-α may also be induced in BMM by CSF-1 (Willman, C., unpublished data). These data thus suggest that the combinatorial effects of the different stimulatory (IFN-γ, GM-CSF, IL-4) and inhibitory (CSF-1, TNF-α) factors produced by resident and infiltrating cells in specific tissue microenvironments may ultimately determine the level of Ia expression in resident macrophages, thereby regulating the ability of the macrophage in that tissue to present antigen and initiate the immune response.

Summary

CSF-1 and granulocyte/monocyte CSF (GM-CSF) were shown to modulate the levels of Ia gene and protein expression in bone marrow–derived macrophages (BMM). Recombinant GM-CSF induced high levels of Ia expression, similar to the levels induced by INF-γ, while IL-3 had no effect. In contrast, recombinant CSF-1 not

only suppressed the basal levels of Ia gene and protein expression in BMM, but also inhibited the induction of Ia by IFN-γ and GM-CSF. Basal levels of Ia were not inhibited by recombinant CSF-1 until after 16–24 h of culture, suggesting an indirect mechanism of suppression. IFN-α/β and PGE_2 were shown not to be involved in the CSF-1 inhibition of basal levels of Ia expression. However, the CSF-1-mediated suppression of both the basal levels of Ia expression and the induction of Ia in BMM by IFN-γ and GM-CSF did correlate with the induction of cellular proliferation. These data imply that in addition to regulating hematopoiesis, CSFs may regulate the initiation of the immune response through their effects on Ia expression in macrophages.

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EXHIBIT 4

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Differential Production of IFN-α/β by CSF-1- and GM-CSF-Derived Macrophages

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Mature macrophages, derived in vitro from bone marrow progenitors under the influence of either macrophage colony stimulating factor (CSF-1) or granulocyte-macrophage (GM)-CSF, have been shown to differ morphologically and functionally. The data presented in this report demonstrate that macrophages derived from bone marrow progenitors under the influence of CSF-1 are highly resistant to infection with vesicular stomatitis virus (VSV), and that this refractoriness can be reversed by treatment of cells with anti-IFN-α/β antibody. In contrast, macrophages derived from bone marrow progenitors under the influence of GM-CSF are highly susceptible to the cytopathic effects of VSV, but can be protected by very low concentrations of exogenous IFN-α/β. These findings suggest that CSF-1 derived macrophages have a greater capacity for the production and/or utilization of IFN-α/β than GM-CSF-derived macrophages, which may account for many of the differentiative differences reported previously.

Key words: GM-CSF, CSF-1, virus sensitivity

INTRODUCTION

Macrophages are a ubiquitously distributed, heterogeneous population of fixed and circulating mononuclear phagocytes. Morphological, functional, and metabolic differences reflect macrophages at different points within a "differentiative hierarchy", as they mature in response to cell-derived and environmental signals [1]. One such group of cell-derived soluble factors (or cytokines) are the colony stimulating factors (CSF). Both fibroblast-derived, macrophage colony stimulating factor (CSF-1) and T-cell-derived, granulocyte-macrophage colony stimulating factor (GM-CSF) have been shown to give rise to mature macrophages in vitro from bone marrow progenitors [15]. Studies from ours and other laboratories have demonstrated that mature macrophages derived in vitro from bone marrow progenitors under the influence of either CSF-1 or GM-CSF differ morphologically and functionally. Specifically, CSF-1-derived macrophages are "more differentiated" with respect to size and phagocytic capacity (both latex ingestion and Fc receptor-mediated phagocytosis) [3], whereas GM-CSF-derived macrophages exhibit high basal levels of Ia antigen expression and an augmented capacity to stimulate antigen-specific T cell proliferation [4,5], as well as a greater potential for tumoricidal activity [2]. Thus, heterogeneity among populations of mature macrophages [11] may reflect the effects of different colony stimulating factors on bone marrow progenitors and/or the emigration of selected populations to particular organs.

Previous studies have indicated that the interferons also represent an important class of cytokines with potent macrophage differentiating effects. Studies by Moore et al. [16] support the hypothesis that bone marrow progenitors stimulated by CSF-1 produce IFN-α/β during the course of their expansion, and that this endogenous product can be used as an autocrine differentiation signal [17,23]. IFN-α/β has also been shown to increase macrophage size and spreading [18], as well as to augment and/or sustain macrophage Fc receptor expression [20].

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This paper represents work done in partial fulfillment of the requirements for the Ph.D. degree.

Given our earlier observation that CSF-1-derived macrophages are larger and more phagocytic than GM-CSF-derived macrophages, we sought to compare production of IFN-α/β by these two populations. The data presented herein support the hypothesis that functional differences between CSF-1- and GM-CSF-derived macrophages can be related, in part, to differential production of IFN-α/β.

MATERIALS AND METHODS

Mice

C3H/HeJ mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and were housed as described previously [9].

Reagents

Murine CSF-1 was purified exactly as described previously [3]. Briefly, serum-free supernatants from L929 fibroblasts were harvested, concentrated, and subjected to a series of chromatographic purification steps which included hydroxylapatite, DEAE Sephacel, Affi-gel 202, and C-18 reverse-phase chromatography. Recombinant human CSF-1 (rCSF-1) was generously provided by Cetus Corp. (Emeryville, CA; specific activity $> 5 \times 10^7$ U/mg). Recombinant GM-CSF (rGM-CSF) was generously provided by Immunex Corp. (Seattle, WA; specific activity $= 4 \times 10^7$ U/mg) and Biogen SA (Geneva, Switzerland; specific activity $= 1.2 \times 10^7$ U/mg). IFN-α/β (specific activity $> 5 \times 10^8$ U/mg) was generously provided by Dr. M. Pauker (Medical College of Pennsylvania, Philadelphia, PA). The NIH Murine Interferon Standard (Reference Reagent No. G-002-904-511), anti-murine IFN-α/β antibody (NIH Reference Reagent No. G-024-501-568), and the control antibody (NIH Reference Reagent No. G-025-501-568) were obtained from the Antiviral Research Branch (NIAID, Bethesda, MD).

Bone Marrow Culturing Techniques

Bone marrow progenitors were cultured in liquid culture in the presence of rGM-CSF or CSF-1 (natural or recombinant) precisely as described previously [3]. Briefly, bone marrow cells were centrifuged on Lymphocyte Separation Medium (LSM; Litton Bionetics, Charleston, SC). Cells harvested from the interface were cultured at 1×10^7 cells/flask (for CSF-1) or 3×10^7 cells/flask (for rGM-CSF) in 10 ml of supplemented Eagle's minimal essential medium (EMEM; M.A. Bioproducts) with 10% fetal calf serum (Hyclone, Logan, UT) which contained 250 U/ml of the rGM-CSF or CSF-1 (day 0). After 24 hr (day 1), nonadherent cells from each flask were transferred to a second flask and on Days 1 and 4 were supplemented with an additional 10 ml of medium which contained 250 U/ml of either rGM-CSF or CSF-1. After 7 days in culture, the macrophages were removed enzymatically and resuspended in RPMI 1640 (M.A. Bioproducts) supplemented with 2% FCS and other additives as described elsewhere [3]. Macrophages were re-plated into 96-well plates (Falcon Plastics, Oxnard, CA) at 2×10^5/well in the *absence* of exogenous colony stimulating factors.

Sensitivity of GM-CSF- and CSF-1-Derived Macrophages to Vesicular Stomatitis Virus (VSV)

Colony stimulating factor-derived macrophages were re-cultured on day 7 in 96-well plates at 2×10^5 macrophages per well. The cells were allowed to adhere for 6 hr. At this time, supernatants were gently aspirated and 0.1 ml of a vesicular stomatitis virus (VSV) suspension [Indiana strain; multiplicity of infection (MOI) of 0.1 or 1.0], diluted in medium, was added to each well. Macrophage cultures were infected for 24 hr, at which time the supernatants were removed and frozen at −70°C for subsequent analysis of virus yield. The adherent cells were washed to remove cellular debris and then fixed for 10 min with 5% formaldehyde and stained for 10 min with 0.05% crystal violet to demonstrate cytopathic effect (CPE). For certain experiments, cultures were treated with either serial twofold dilutions of IFN-α/β or antibody for 24 hr prior to infection with VSV at an MOI $= 0.1$. Fifty percent CPE was determined spectrophotometrically as described elsewhere [19].

Quantitation of Virus Production in rGM-CSF- Versus CSF-1-Derived Macrophages

Culture supernatants from virus-infected macrophages were examined for viral replication as described by Vogel and Fertsch [21]. Briefly, L929 fibroblasts were grown to confluency in six-well tissue culture plates (Falcon). Cells were infected for 1 hr at room temperature with 1.0 ml of virus sample diluted in EMEM-10% FCS. After the 1-hr adsorption period, the contents of each well were removed and the monolayer overlaid with 1.0 ml of phenol red-free EMEM which was supplemented with sodium bicarbonate, glutamine, penicillin and streptomycin, 5% FCS, and 1% Noble agar (Difco Laboratories; Detroit, MI). Once solidified, the cultures were incubated at 32°C for 2 days in 5% CO_2. Plaques were developed by overlaying each culture with 1 ml phenol red-free EMEM supplemented as described above, but with the addition of neutral red dye (GIBCO Laboratories, Grand Island, NY; 1:150 dilution). Cultures were incubated at 37°C for 4–6 hr and then scored visually for viral plaque formation. Each supernatant was assayed at multiple dilutions in duplicate.

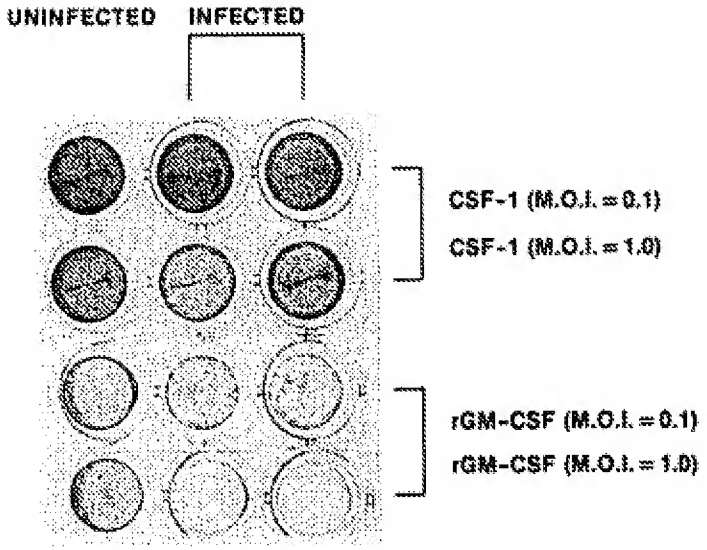

Fig. 1. Sensitivity of bone marrow-derived macrophages to infection with vesicular stomatitis virus (VSV). C3H/HeJ bone marrow progenitors were cultured for 7 days in liquid culture in the presence of CSF-1 or rGM-CSF as described in the Materials and Methods. Following this 7 day culture period, mature macrophages were harvested and re-plated (in the absence of CSF) at a cell concentration of 2 × 10^5 cells per well in 96-well culture plates. The macrophages were allowed to adhere for 4–6 hr. Following this adherence step, the supernatants were removed and 0.1 ml of VSV suspension added at an MOI of 0.1 or 1.0. Twenty-four hours postinfection, the supernatants were removed and each monolayer was fixed, stained, and examined visually and spectrophotometrically for virus-induced CPE. These results were derived from a single experiment representative of eight separate experiments.

Fluorescent Analysis of rGM-CSF- and CSF-1-Derived Macrophages Following VSV Infection

To detect intracellular VSV in infected cells, rGM-CSF- or CSF-1-derived macrophages were re-plated at 2 × 10^5 per well in 8-well Lab-Tek tissue culture chambers (Miles Laboratories, Inc., Naperville, IL) and were allowed to adhere for 4–6 hr. Following this adherence stage, culture supernatants were removed and 0.1 ml of VSV suspension, diluted in medium (MOI of 0.1), was added to each well. Cultures were infected for 6 hr, acetone-fixed for 10 min, and the slides frozen at −20°C. Prior to fluorescent staining, culture slides were re-hydrated with 0.1 ml of a solution which contained phosphate buffered saline (PBS), 1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO), and 5% goat serum (GS; GIBCO Laboratories) for 15 min at room temperature. Cultures were washed with PBS/BSA/GS and incubated with rabbit anti-VSV antibody (Lee Biomolecular Research Inc., San Diego, CA) diluted 1:50 in PBS/BSA/GS for 30 min at room temperature. At a final concentration of 1:10,000 in a total volume of 0.1 ml, this preparation of anti-VSV antibody was found to inhibit CPE in L929 fibroblasts infected with VSV (MOI = 0.1). Prior to addition of a fluorescent secondary antibody, cultures were subjected to three 15 min washes with PBS/BSA/GS. Cultures were then incubated with 0.05 ml of rhodamine-conjugated, goat anti-rabbit IgG antibody (Cooper Biomedical Inc., Malvern, PA) diluted 1:50 in PBS/BSA/GS for 30 min at 37°C in the dark. Cultures were washed for two 15 min periods and one 30 min period using PBS/BSA/GS (in the dark). Cultures were examined for specific fluorescence using an Olympus BH-2 epi-fluorescent microscope (40× magnification). Control cultures consisted of i) uninfected macrophage cultures treated with the anti-VSV antibody and rhodamine-conjugated secondary antibody and ii) VSV-infected cultures treated with rhodamine-conjugated secondary antibody only.

RESULTS

Sensitivity of Bone Marrow-Derived Macrophages to Vesicular Stomatitis Virus (VSV)

Interferons are probably best recognized for their capacity to mediate an antiviral state in interferon-sensitive cell types [reviewed in 7]. Therefore, we compared CSF-1 and GM-CSF-derived macrophages for the ability to resist virus infection as an indirect measure of endogenous interferon production. Figure 1 shows that C3H/HeJ macrophages derived from BMP under the influence of highly purified CSF-1 were resistant to the cytopathic

effects (CPE) of VSV when infected at an MOI of 0.1. When the MOI was increased tenfold, CSF-1-derived macrophages exhibited only minimal sensitivity, as evidenced by slightly less uptake of crystal violet by the monolayers. By comparison, rGM-CSF-derived macrophages were much more sensitive to VSV-induced CPE at both an MOI of 0.1 and 1.0. The difference in crystal violet intensity observed in uninfected rGM-CSF- versus uninfected CSF-1-derived macrophages is due to an inherent inability of rGM-CSF-derived cells to incorporate the crystal violet and not due to differences in cell densities. For example, in a single representative experiment, the OD_{595} associated with uninfected CSF-1-derived macrophages was 0.312 whereas, the OD_{595} for uninfected GM-CSF-derived macrophages was 0.146. In contrast, the cell densities of GM-CSF- and CSF-1-derived monolayers were verified repeatedly by quantitation of total cell protein by the Lowry method and found to be identical [13]. Specifically, in three separate experiments, the protein concentration of cells derived in the presence of CSF-1 versus GM-CSF were 26.0 ± 1.0 and 27.6 ± 1.4 μg per 2×10^5 macrophages, respectively. To quantify the CPE observed in bone marrow-derived macrophage cultures, uninfected monolayers were compared to infected cultures and the percent CPE determined by the amount of crystal violet stain taken up by the remaining intact cells [19,22]. A comparison of infected to uninfected monolayers for each macrophage population showed that while CSF-1-derived macrophages exhibited 1 ± 1% CPE, rGM-CSF-derived macrophages exhibited 71 ± 7% CPE for 8 separate experiments in cultures infected with an MOI of 0.1.

Although all of our studies thus far utilized the endotoxin hyporesponsive (Lps^d) C3H/HeJ mouse strain, differences in VSV sensitivity were also observed if the macrophages were derived from the syngeneic, but endotoxin-responsive (Lps^n), C3H/OuJ strain. However, the CPE induced in C3H/OuJ macrophages derived under the influence of rGM-CSF was somewhat less striking (34 ± 11% for n = 5 separate experiments). As was observed with CSF-1-derived, C3H/HeJ macrophages, C3H/OuJ macrophages derived under the influence of CSF-1 were completely refractory to VSV-induced CPE.

Fluorescent Analysis of VSV Infectivity in Bone Marrow-Derived Macrophages

To ensure that both cell types were equally capable of being infected with VSV, the macrophages were compared 6 hr postinfection for the presence of intracellular VSV as detected by fluorescent antibody staining. Table 1 shows that there was no significant difference in the ability of rGM-CSF-derived macrophages versus CSF-1-derived macrophages to be infected with VSV.; i.e., 11.3% of infected rGM-CSF-derived macrophages and 16.5% of the CSF-1-derived macrophages stained positively for VSV proteins 6 hr postinfection. These figures are consistent with an estimated MOI = 0.1. Therefore, the failure of VSV-infected CSF-1-derived macrophages to exhibit CPE was not related to an inability of these cells to be infected.

TABLE 1. Fluorescent Analysis of VSV Infectivity in Bone Marrow-Derived Macrophages[a]

Macrophage type	Percent fluorescent[b]
GM-CSF-derived	11.3 ± 2.5
CSF-1-derived	16.5 ± 4.2

[a]Two × 10^5 mature D7 CSF-1- or GM-CSF-derived macrophages were cultured as described in Materials and Methods and allowed to adhere for 4–6 hr. Following this adherence step, macrophage supernatants were removed and replaced with medium alone or 0.1 ml of VSV at an MOI = 0.1. Culture supernatants were removed 6 hr later, at which time monolayers were acetone-fixed for subsequent detection of viral protein production using fluorescent antibody analysis as described in the Materials and Methods.

[b]Results are from a single representative experiment and were determined based on the No. of fluorescent (positive) cells in a field (approximately 80 macrophages/field). The No. of cells per field were comparable for all treatment groups based on phase contrast microscopy. The data represent the mean ± standard error of the mean for four separate fields for each macrophage type. Control cultures showed no detectable fluorescence.

Comparison of Viral Replication in rGM-CSF- and CSF-1-Derived Macrophages

To address the mechanism by which VSV induced CPE in rGM-CSF-derived macrophages, we compared VSV replication within these two macrophage populations. Culture supernatants were collected 24 hour postinfection from VSV-infected macrophages and were assayed for virus yield. Table 2 shows that no significant difference was observed in the virus yields between rGM-CSF- and CSF-1-infected macrophages. Since the macrophage cultures were infected at an MOI = 0.1 (i.e., with 2×10^4 VSV plaque forming units (PFU)/well or 2×10^5 PFU/ml), the resultant plaque data derived from the supernatants of infected cells indicate that no significant viral replication occurred. Therefore, increased viral replication in rGM-CSF-derived macrophages cannot account for the differential CPE observed between the two cell types.

Endogenous Interferon Production in rGM-CSF- and CSF-1- Derived Macrophages

The role of interferon in antiviral protection has been firmly established [reviewed in 6 and 7]. In addition, the ability of macrophages to produce IFN-α/β has also been well-documented [8,16,20,23]. Therefore, the potential role of endogenous IFN-α/β production as a possible mediator of the resistance to VSV-induced CPE in

TABLE 2. Plaque Formation in Virus-Infected GM-CSF- and CSF-1-Derived Macrophage Culture Supernatants*

Macrophage type	Virus yield (PFU/ml)[b]
GM-CSF-derived	4.2×10^5 [1.1×10^5–1.5×10^6]
CSF-1-derived	2.8×10^8 [5.0×10^4–1.5×10^8]

*Two $\times 10^5$ rGM-CSF- and CSF-1-derived macrophages were cultured in 96-well tissue culture plates as described in Figure 1. Macrophages were incubated for 4–6 hr, at which time the culture supernatants were replaced with 0.1 ml of VSV suspension (MOI = 0.1). Macrophages were infected for 24 hr and the virus supernatants removed and quantified for the No. of infectious particles in a plaque forming assay.

[b]The data represent the geometric means derived from seven separate experiments for rGM-CSF-derived macrophages and four separate experiments for CSF-1-derived macrophages. The 95% confidence limits for the treatment groups are indicated in brackets.

CSF-1-derived macrophages was next examined. Initially, we sought to measure IFN activity directly in the supernatants of GM-CSF and CSF-1-derived macrophage cultures. However, the levels of antiviral activity which were detectable in the GM-CSF-derived supernatants were always below the lower limit of the standard antiviral assay [~3 U/ml; 19,22], and the levels in CSF-1-derived macrophage supernatants were at the lower limits of detection within the assay (data not shown). Therefore, we sought a more sensitive method for demonstrating differences in interferon production by rGM-CSF- and CSF-1-derived C3H/HeJ macrophages. VSV-sensitive, GM-CSF-derived macrophages were cultured as described in Table 2, and treated for 24 hr with serial two-fold dilutions of the NIH Reference Reagent, murine IFN-α/β to determine the minimal amount of IFN-α/β required to protect these macrophages from VSV-induced CPE. rGM-CSF-derived macrophages were half-maximally protected from VSV-induced CPE by pretreatment of cultures with 1.0 ± 0.2 U/ml of exogenous IFN-α/β (n = 6 separate experiments). In contrast, the VSV-resistant, CSF-1-derived macrophages were treated with serial twofold dilutions of the NIH Reference Reagent, anti-murine IFN-α/β antibody, in an attempt to demonstrate that the protective factor in CSF-1-derived macrophages was endogenously-produced IFN-α/β. Inclusion of anti-IFN-α/β antibody, but not the control antibody, 24 hr prior to VSV infection of CSF-1-derived macrophages resulted in reversal of the refractoriness of these cultures to VSV infection. Reversal of resistance to 50% CPE was observed in these cultures following treatment with a 1:182 dilution of the NIH Reference Reagent anti-murine IFN-α/β (titer = 182 with 95% confidence limits of 93.5–355; n = 9 separate experiments). However, the control antibody (NIH Reference Reagent No. G-025-051-568) failed to reverse the refractoriness to VSV, even at the highest concentration tested (1:20). Therefore, the difference observed between CSF-1- and rGM-CSF-derived macrophages with respect to sensitivity to VSV infection can be related to differences in endogenous IFN-α/β production by the two populations. rGM-CSF-derived macrophages exhibit lower levels of endogenous IFN-α/β in contrast to the levels exhibited by CSF-1-derived C3H/HeJ macrophages.

DISCUSSION

A role for endogenous IFN production by macrophages and the modulation of macrophage differentiation in response to this autocrine signal has been well-documented. Specifically, Fc receptor expression, as well as Fc-mediated phagocytosis [16,20,23], Mac-1 antigen expression [20], and Ia antigen expression in neonatal macrophages [10] all appear to be regulated by IFN-α/β in an autocrine manner. In addition, previous studies have shown that macrophages derived from the bone marrow under the influence of CSF-1 exhibit increased latex phagocytosis, Fc receptor expression, and Fc-mediated phagocytosis when compared to those derived under the influence of GM-CSF [3]. Based on these findings, we hypothesized that perhaps a major difference between these two populations of macrophages resides in the ability to produce IFN-α/β endogenously. Examination of the endogenous IFN-α/β production by these two populations would provide direct support for this theory. However, the conventional antiviral assay used to measure secreted IFN activity was found not to be adequately sensitive to test this hypothesis directly. Based on the well-documented role of IFN in antiviral activity [reviewed in 7], GM-CSF- and CSF-1-derived macrophages were compared for sensitivity or resistance to viral infection. The observed viral sensitivity of GM-CSF-derived macrophages versus the refractoriness of CSF-1-derived macrophages to VSV infection further supported the possibility that differences in IFN production between these two macrophage populations might exist. Confirmation of this theory was obtained following reversal of the GM-CSF-derived macrophage's VSV-sensitive phenotype by exogenous addition of IFN-α/β and by reversal of the VSV-resistant phenotype of CSF-1-derived macrophages by addition of anti-IFN-α/β antibodies. These findings strongly support a role of IFN-α/β in differential viral susceptibility of these two macrophage populations. Moreover, these findings support the hypothesis that there is a differential ability of GM-CSF- and CSF-1-derived macrophages to produce IFN-α/β endogenously. However, the mechanism by which VSV induces CPE in GM-CSF-derived macro-

phages was not elucidated in this study. Since neither GM-CSF- nor CSF-1-derived macrophages supported any significant viral replication (Table 2), it is unlikely that viral replication resulting in the release of infectious viral particles mediated the observed CPE in the GM-CSF-derived macrophage cultures. These findings are similar to those of McGowan and Wagner [14], which demonstrated that virus production was not a prerequisite for VSV-induced CPE. In addition, the observation that both populations of macrophages were comparably infected (Table 1) indicates that the CPE observed in GM-CSF-derived cultures may be attributable to differences in the maturation of viral progeny late in culture.

The mechanisms by which CSF-1 and GM-CSF differ with respect to the induction of endogenous IFN-α/β production in macrophages is yet to be established. One possible explanation supported by the data shown in this report is that CSF-1 is a better inducer of endogenous IFN during macrophage differentiation than GM-CSF. This possibility is consistent with work carried out by Lee and Warren [12] in studies where mature murine peritoneal macrophages were treated with 1,000–2,000 U/ml of CSF-1 or GM-CSF and then infected with VSV at an MOI of 2.0. They found that CSF-1 could induce resistance to VSV infection, as evidenced by reduced viral yield, and that this resistance was reversed by anti-IFN-α/β antibody. Our failure to detect VSV progeny in GM-CSF-derived macrophages, in contrast to increased viral yield seen by Lee and Warren, may be related to i) the cell type (i.e., peritoneal exudate versus bone marrow-derived macrophages), ii) the indicator cells used in their plaque assay, iii) differences in CSF concentration, or iv) VSV MOI.

An alternate explanation for our findings might be that GM-CSF blocks induction of endogenous IFN-α/β and/or precludes macrophages from responding to exogenous addition of IFN-α/β. However, the latter possibility is highly unlikely since GM-CSF-derived macrophages were found in this study to respond to as little as 1.0 U/ml exogenous IFN-α/β to exhibit an antiviral state.

In conclusion, these studies demonstrate the differential ability of bone marrow progenitors stimulated by GM-CSF or CSF-1 to result in the maturation of macrophages with different capacities for IFN-α/β production. These findings add to a growing body of evidence that the expansion of a mature macrophage population from progenitors under the influence of distinct colony stimulating factors may contribute in a significant way to heterogeneity exhibited among mature macrophage populations. These findings also illustrate the potential role of secondary mediators produced in response to colony stimulating factors as part of a complex cytokine cascade which leads to the propagation and differentiation of macrophages.

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EXHIBIT 5

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Bone marrow-derived macrophages grown in GM-CSF or M-CSF differ in their ability to produce IL-12 and to induce IFN-γ production after stimulation with *Trypanosoma cruzi* antigens

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Abstract

Trypanosoma cruzi is the etiological agent of Chagas' disease in man. Control of parasitism at the beginning of experimental infection depends on cytokine-activated macrophages that synthesize nitric oxide (NO). We investigated macrophage populations derived in the presence of M-CSF (M-MØ) or GM-CSF (GM-MØ) regarding their ability to control intracellular parasitism by *T. cruzi* and to synthesize IL-12 and NO. Both macrophage populations supported intracellular multiplication of the parasite; when activated by IFN-γ, GM-MØ exerted better control of parasitism. Stimulation of GM-MØ with *T. cruzi* or *Staphylococcus aureus* resulted in IL-12 production and higher levels of NO synthesis in comparison with stimulated M-MØ. Mice immunized with parasite-Ag-pulsed GM-MØ but not with pulsed M-MØ had increased IFN-γ and IL-2 production in lymph nodes. However, when immunization was followed by infection with live parasites, transient elevation of IFN-γ production was observed in both GM-MØ- and M-MØ-immunized mice, without reduction of blood parasite levels. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Macrophages populations; Nitric oxide; Cytokines; GM-CSF; M-CSF; Chagas' disease

1. Introduction

Infection with the protozoan parasite *Trypanosoma cruzi* results in a life-long infection, which can lead to Chagas' disease, a debilitating illness affecting heart or digestive function in 30% of chronic phase-patients.

T. cruzi can infect macrophages amongst a variety of other host cell types; intracellular replication occurs as amastigotes followed by the release of trypomastigotes that can be carried by the bloodstream to infect all organs.

Control of *T. cruzi* parasitism during the first weeks of infection is considered to be critically dependent on effective macrophage activation by cytokines. Evidence has accumulated showing that the addition of IFN-γ [1–4], GM-CSF [5,6] or TNF-α [4,5] to cultures of *T. cruzi*-infected macrophages results in more efficient killing of amastigotes by the phagocytes. The in vivo administration of IFN-γ [7], TNF-α [8] or IL-12 [9] early during infection effectively reduces blood parasitism and mortality.

Intracellular killing of *T. cruzi* by IFN-γ- or TNF-α -activated macrophages is mediated largely by nitric oxide [4]. IFN-γ synthesis at the initial phase of infection is IL-12 dependent [10,11] and important for the control of *T. cruzi* infection by innate [9] and acquired immunity [10,11].

In the murine model of *Leishmania major* infection, immunization with parasite-antigen pulsed macrophages grown in GM-CSF (GM-MØ), but not with similarly pulsed macrophages grown in M-CSF (M-MØ), stimulates IFN-γ production in draining lymph nodes (LN) and increases the resistance of susceptible BALB/c mice to infection [12]. This study indicates that the type of macrophage that interacts with antigen (Ag) in vivo may influence the immune

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response by creating a *milieu* favorable to the development of an early protective cytokine response. More recently, the same authors correlated the observed ability of Ag-pulsed GM-MØ to locally induce IFN-γ, to increased levels of IL-12 expression in the target LN [13].

Those two populations of macrophages also differ regarding other important parameters of the immune response. In comparison with M-MØ, GM-MØ express higher levels of MHC molecules [14], are more efficient at antigenic presentation and Th1 cell stimulation [15], exhibit enhanced tumoricidal and microbicidal activity [16] and produce more NO [17].

It has been suggested that earlier and higher IFN-γ production by *T. cruzi* infected mice is an important factor for their resistance to infection [18,19]. Thus, considering the potential of Ag-pulsed GM-MØ macrophages to enhance IFN-γ production at the beginning of infection, the utilization of these cells to potentiate immunity to *T. cruzi* antigens and to infection becomes interesting. In addition, it allows a better understanding of the differential activation of macrophage populations by GM-CSF and M-CSF.

In this paper we show that *T. cruzi* or *Staphylococcus aureus* (SaC) stimulated GM-MØ in comparison with stimulated M-MØ produced IL-12 and higher levels of NO. Immunization of BALB/c mice with *T. cruzi* Ag-pulsed GM-MØ led to enhanced transient IFN-γ production by draining LN cells. However, when challenged with live parasites, GM-MØ as well as M-MØ immunized mice showed enhanced IFN-γ production but neither group was protected against the infection.

2. Materials and methods

2.1. Animals

Female SPF BALB/c mice were obtained from the breeding animal facilities of the Department of Immunology, ICB, USP. Mice were used at 6–9 weeks of age. All procedures with the animals were in accordance with the principles of the Brazilian Code for the Use of Laboratory Animals.

2.2. Parasite Ag preparation and parasite infection

Trypanosoma cruzi (Y strain) tissue culture trypomastigotes were obtained from supernatants of infected LLC-MK2 cells (ATCC CCL7.1) as already described [20]. Briefly, LLC-MK2 cells were maintained in culture flasks with DMEM containing 2 mM glutamine, 63 mg/l penicillin, 100 mg/l streptomycin sulfate (Sigma Chemical Co., St. Louis, MO, USA) and 5% fetal calf serum (FCS-Guanandhy, Cuiabá, MT, Brazil). Subconfluent cultures were infected with *T. cruzi* trypomastigotes and the supernatants were collected daily between days 5 and 9 after infection, washed three times in sterile phosphate buffer saline (PBS) (1900 g, 20 min, 4°C) and resuspended at 10^8 or 10^9 parasites per ml. Each parasite sample was frozen and thawed ten times to prepare *T. cruzi* antigen (Tc-Ag) and stored at −20°C.

T. cruzi (Y strain) was maintained by weekly intraperitoneally (i.p.) injection into BALB/c mice. The parasite-rich blood was appropriately diluted in 0.01 M sterile PBS to contain 1000 blood trypomastigotes in 200 μl and injected subcutaneously (s.c.) in mice at both sides of the base of the tail.

2.3. Obtaining GM-CSF and M-CSF grown macrophages

The methods used to prepare these two macrophage populations have been described in detail [12]. Briefly, single cell suspensions of bone marrow cells were cultured in 6-well culture dishes at 2×10^6 cells/ml in RPMI medium (Sigma Chemical Co.), supplemented with 2 mM glutamine, 63 mg/l penicillin, 100 mg/l streptomycin sulfate, 50 μM 2-ME and 10% FCS containing either 40 ng/ml murine recombinant GM-CSF (a gift from Dr Robert Coffman, DNAX Research Institute, Palo Alto, CA) or 30% (v/v) L929 cell-conditioned medium as a source of M-CSF [12]. The cultures were rinsed and medium was replenished every 3 days in order to remove non-adherent cells. Adherent cells were used in the experiments on day 10 after initiation of the cultures. Adherent cells were of homogeneous morphology, consisting of mononuclear cells with abundant cytoplasm. There were no cells with doughnut-shaped nucleus or polymorphonuclear cells. In order to characterize the cell populations present in the culture, cell suspensions from 10-day-old cultures were stained by fluorescence after treatment with Fc receptor-blocking Ab 2.4G2 (20 μg per well) and analyzed by Flow Cytometry (Becton Dickinson, San Diego, CA). Cells were stained with FITC-anti-CD11b (Mac-1) and PE-anti-F4/80, which are markers preferentially expressed in macrophages. Double staining with PE-anti-CD11c (clone N418) and FITC-anti-CD8α was performed to determine the frequency of dendritic cells (DC) expressing the CD11c marker and the percentage of DC expressing CD8α considered a marker of DC of lymphoid origin [21]. Antibodies were purchased from Pharmingen, San Diego, CA. Cultures of M-MØ stained positive for CD11b (Mac-1) at 86.1% and at 71.4% for F4/80 whereas for GM-MØ cultures, the percentages were, respectively, 92.6% and 73.1%. The percentages of myeloid DC ($CD11c^+/CD8\alpha^-$ cells) were: 20.1% (M-MØ cultures) and 11.6% (GM-MØ cultures); 'lymphoid' DC ($CD11c^+/CD8\alpha^+$ cells) per-

centages were 4.6% (M-MØ cultures) and 2.5% (GM-MØ cultures).

2.4. In vitro macrophage infection

Cultures of GM-CSF-grown macrophages (GM-MØ) or M-CSF-grown macrophages (M-MØ) were dispensed (2×10^5) into glass chamber slides (four chambers to a slide) (Nunc, Naperville, IL), and infected with *T. cruzi* at 8:1 parasite:cell ratio for 6 h at 37°C in 7% CO_2. After that, cultures were washed to remove extracellular parasites and half the slides were stained with HEMA 3 stain set (Biochemical Sciences, Swedesboro, NJ). The remaining washed cultures were further incubated in complete RPMI medium for 42 h. After this period, the supernatants were collected and the slides were washed and stained with HEMA 3. The harvested supernatants were used to measure IL-12, IL-10 and NO levels. Some cultures were treated with murine rIL-4 (10 ng/ml), murine rIFN-γ (100 U/ml) (gifts from Dr Robert Coffman, DNAX Institute), rat anti-murine IL-10 mAb 2A5 (20 μg/ml) and indomethacin (20 μg/ml-Sigma Chemical Co.) as an inhibitor of prostaglandin (PG) synthesis [22] in order to evaluate modulation of NO or IL-12 production. These reagents were added at the beginning of the cultures and left for the duration of the cultures (48 h); for the cultures infected with *T. cruzi*, the reagents were added at time zero and added again at 6 h after the removal of extracellular parasites.

2.5. Immunization of mice with macrophages and infection

Bone marrow-derived macrophages (GM-MØ or M-MØ) were incubated in 6-well culture dishes (overnight at 37°C in CO_2) with Tc-Ag at 500 μg/ml (equivalent to 10^8 parasites per ml). After this incubation period, the cultures were washed two times with PBS, resuspended in EDTA 0.1% in PBS and incubated at 37°C for 30 min. Complete RPMI medium was then added and the cells were gently scrapped from the plastic surface, washed twice and resuspended in RPMI medium. Mice received injections of 6×10^6 macrophages at the base of the tail, 50 μl at each side. As control groups, some mice received 100 μg of Tc-Ag. Groups of mice were also infected with 1000 *T. cruzi* blood forms, 24 h after immunization with Ag-pulsed macrophages or with Tc-Ag, by the same route.

2.6. Lymph node cell cultures

Mice were killed 5 and 8 days after immunization and the superficial inguinal and abdominal periaortic lymph nodes were removed and LN cell suspensions prepared. The cells were cultured in complete RPMI medium in 96-well flat-bottomed plates at a density of 6×10^5 cells/200 μl per well for proliferation assays or in 24-well plates at 10^7 cells/ml or at 3×10^6 cells/ml to harvester supernatants at 24 or 72 h of culture, respectively. The cultures were stimulated with Tc-Ag (corresponding to 1.2×10^6 parasites per well). [^{3}H]-TdR (0.5 μCi per well) was added to the proliferation assays 18 h before cell harvesting at 72 h of culture. Radioactivity was measured by scintillation counting and the data presented as the arithmetic mean (cpm) of triplicate cultures and standard deviation (S.D.).

2.7. Cytokine assays

Cytokine levels in LN culture supernatants (24 h for IL-2 and IL-4 measurements and 72 h for IFN-γ and IL-10, in LN cultures) and IL-12 in macrophage culture supernatants (48 h) were measured by two-site sandwich enzyme-linked immunosorbent assay (ELISA). The following mAb pairs were used of which the second cited was biotinylated: IFN-γ, XMG 1.2 and AN 18; IL-2, 1A12 and 5H4; IL-10, JES-2A5 and SXC-1; IL-4, 11B11 and BVD624G2; IL-12 (p40), C17.15 and C15.6. Standard curves were obtained with recombinant mouse cytokines. The minimal detectable concentration in each test is: IFN-γ, 1.6 ng/ml; IL-2, 0.4 ng/ml; IL-10, 3.1 U/ml; IL-4, 0.16 ng/ml; IL-12, 0.31ng/ml. The reaction was developed with peroxidase-conjugated streptavidin followed by the substrate mixture containing hydrogen peroxide and ABTS as chromogen. The supernatants were tested in serial two-fold dilutions and the results expressed as the arithmetic mean of duplicate determinations ± standard error of the mean (S.E.M.).

2.8. NO determinations

The nitrite content in duplicate serial diluted samples was measured by adding 50 μl of freshly prepared Griess reagent [23] to 50 μl of the samples in 96-well plates. Optical densities (OD) were read at 550 nm, 10 min later, by comparison with the OD curves of serial dilutions of sodium nitrite in complete culture medium; minimal detectable concentration was 1.6 μM. The results are expressed as the arithmetic mean of duplicate determinations ± S.E.M.

2.9. Parasitemia levels

To verify the infection progression, parasitemia levels were determined by counting the number of parasites in fresh blood samples [24]. Briefly, 5 μl of blood were collected from a tail vein into a micropipette tip rinsed with anticoagulant (3.8% sodium citrate), and dispensed on a glass slide. After that, a coverslip was placed on the blood sample and the parasites were

counted in 50 fields at 40X magnification. The counted area was extrapolated to the whole area of the coverslip and the results were expressed as number of parasites per ml of blood. The results are expressed as the arithmetic mean of determinations ± S.E.M. for groups of five animals.

2.10. Statistics

Results from macrophage infection experiments and cytokine determinations were analyzed by Tukey–Kramer multiple comparison test. Parasitemia levels were compared by analysis of variance (ANOVA).

3. Results

3.1. Infection and growth of T. cruzi in GM-MØ and M-MØ

Bone marrow-derived macrophages grown in M-CSF or GM-CSF were first compared in their ability to become infected and support intracellular growth of *T. cruzi*. As shown in Fig. 1 A, by 6 h after infection, 98% of M-MØ in the cultures were parasitized; 50% of the total infected cells in culture were heavily parasitized containing more than 10 parasites per cell. In contrast, the percentage of infected GM-MØ was 81% with only 10% of the cells harboring more than 10 parasites. This indicates that *T. cruzi* initial internalization by M-MØ was higher compared with GM-MØ. Following the entry period of 6 h, intracellular multiplication of *T. cruzi* occurs in both GM-MØ and M-MØ, as observed at 48 h by the reduction in the number of macrophages harboring one to five and five to ten parasites accompanied by a parallel increase in macrophages containing more than ten parasites.

We next compared the effect of IFN-γ treatment on the ability of GM-MØ and M-MØ to control intracellular parasitism. Treatment of GM-MØ or M-MØ with rIFN-γ resulted in significant lower infection rates at 6 h after infection as compared with untreated cultures (Fig. 1B vs A). There were no marked differences in the rate of parasitism between M-MØ and GM-MØ treated with rIFN-γ at 6 h (Fig. 1B). After 48 h of culture in the presence of IFN-γ, the total percentage of infected cells was similarly reduced in GM-MØ and M-MØ cultures (Fig. 1B). It should be noted, however, that IFN-γ treated M-MØ still had more heavily parasitized cells (more than six or ten parasites per cell) while none were observed in GM-MØ cultures. Taken together, these results suggest that IFN-γ activated GM-MØ exert a better control of intracellular *T. cruzi* parasitism than similarly activated M-MØ.

3.2. NO production by GM-MØ and M-MØ

As the microbicidal effect of macrophages on *T. cruzi* is dependent on the production of NO by these cells [25], we next investigated possible differences of NO production by GM-MØ and M-MØ. Spontaneous, unstimulated NO production was not significantly different in these macrophage populations and the addition of IFN-γ alone to either type of macrophages did not stimulate NO production (Table 1). Stimulation of GM-MØ or M-MØ cultures with live *T. cruzi* failed to stimulate NO production, whereas stimulation with dead SaC increased NO production only in GM-MØ cultures. Stimulation by either *T. cruzi* or SaC associated to IFN-γ treatment resulted in significant increases of NO production by both types of macrophages, however NO levels measured in GM-MØ cultures were about 100% higher than those seen in M-MØ cultures.

It has been reported that M-MØ when stimulated with IL-4 plus LPS were capable of producing more NO than GM-MØ [15]. As shown in Table 1, IL-4 treatment had an inhibitory effect on NO production

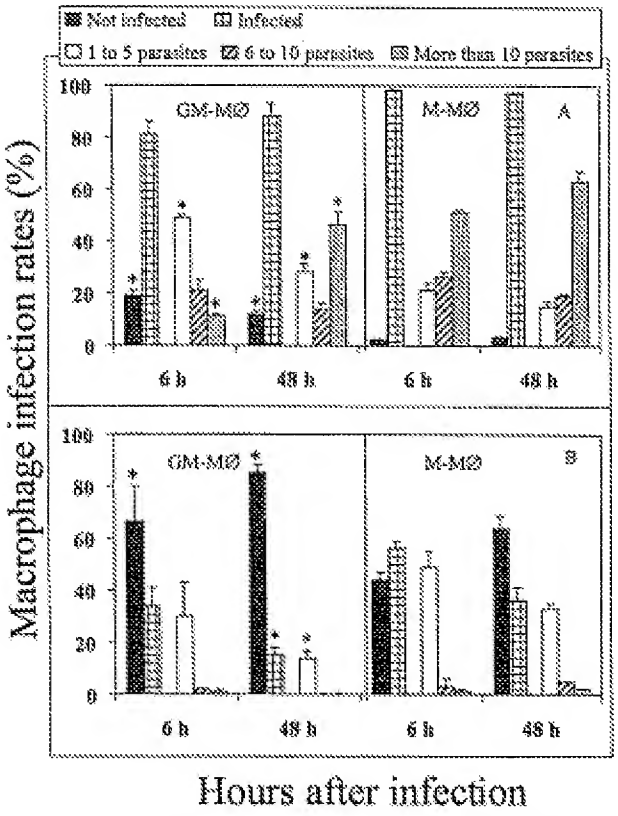

Fig. 1. Infection of GM-CSF (GM-MØ) and M-CSF (M-MØ) grown macrophage cultures with *Trypanosoma cruzi* in the absence (A) or in the presence (B) of rIFN-γ. Results expressed as the mean percentage of non-infected cells, total infected cells and cells harboring one to five, six to ten or more than ten parasites. Means from triplicate cultures ± S.E.M. $^{*}P < 0.05$ GM-MØ vs M-MØ at the same times and conditions of culture. Representative of three experiments.

Table 1
Nitric oxide production by M-MØ or GM-MØ infected with *T. cruzi*

Cells[a]	Stimulus	Nitrite (μM)		
		Medium	IFN-γ (100 U/ml)[b]	IL-4 (10 ng/ml)[b]
M-MØ	–	12.6 ± 2.5	10.6 ± 2.1	7.4 ± 1.5
	SaC	10.2 ± 2.0	28.3 ± 5.7	13.8 ± 3.8
	Live *T. cruzi*	8.3 ± 1.7	23.2 ± 4.6	6.9 ± 1.4
GM-MØ	–	18.2 ± 3.6	17.4 ± 3.5	10.2 ± 2.0
	SaC	38.4 ± 7.7*	62.3 ± 2.5*	11.1 ± 2.2**
	Live *T. cruzi*	21.8 ± 4.4*	42.3 ± 8.5*	10.1 ± 2.0**

[a] Macrophages were obtained as described and cultured in chamber slides with dead *Staphilococcus aureus* strain Cowan (SaC) or infected with *T. cruzi* for 6 h. Supernatants for Nitrite quantification were obtained after 48 h. Means of duplicates ± S.D. Representative of three experiments.

[b] Cytokines were added at the start of the cultures and added again to *T. cruzi*-infected cultures after the initial 6 h period of infection. *$P < 0.05$ compared with identically stimulated and treated M-MØ. **$P < 0.05$ compared with GM-MØ cultures without IL-4-addition.

by *T. cruzi*- or SaC-stimulated GM-MØ cultures, but did not modify NO production by M-MØ cultures.

3.3. IL-12 production by GM-MØ and M-MØ

In order to further characterize the two macrophage populations, we measured IL-12 and IL-10 production by GM-MØ and M-MØ after *T. cruzi*-infection, SaC stimulation or incubation with *T. cruzi* antigen. Cultures were also treated with IL-4, as this cytokine was reported to enhance IL-12 secretion by SaC-stimulated human peripheral blood-derived macrophages [26].

Spontaneous IL-12 production by M-MØ was very low and only low levels were obtained in SaC- or live *T. cruzi*-stimulated cultures (Table 2); treatment with IFN-γ or IL-4 did not augment IL-12 synthesis. In contrast, GM-MØ spontaneously produced higher IL-12 levels that were significantly increased by SaC or live *T. cruzi* stimulation; additional stimulation with IFN-γ did not further increase IL-12 production. Pre-treatment with IL-4 resulted in marked decrease in IL-12 production by GM-MØ.

IL-10 levels were undetectable in unstimulated or stimulated GM-MØ cultures (data not shown) and very low in M-MØ cultures stimulated with SaC (2.78 U/ml), SaC + IFN-γ (5.75 U/ml) and live *T. cruzi* + IFN-γ (8.31 U/ml).

As IL-10 and PG have been described as inhibitors of IL-12 synthesis by macrophages [27], uninfected and *T. cruzi*-infected GM-MØ and M-MØ cultures were treated with neutralizing anti-IL-10 mAb and with Indomethacin, as inhibitor of PG synthesis [22]. No increase of IL-12 synthesis was observed by these treatments (data not shown).

We next asked whether *T. cruzi* antigen (disrupted parasites) could differentially stimulate IL-12 production by cultured GM-MØ and M-MØ. As shown in Table 3, *T. cruzi* antigen pulsing of GM-MØ, but not of M-MØ, stimulated IL-12 production in a dose-dependent way.

3.4. IFN-γ and IL-2 production by draining lymph nodes cell from mice immunized with T. cruzi-Ag-pulsed-GM-MØ or with T. cruzi Ag-pulsed-M-MØ

In order to verify whether the in vitro observed differences between GM-MØ and M-MØ could affect the development of the immune response to parasite antigen in vivo, mice were injected at the base of the tail with GM-MØ or M-MØ earlier incubated overnight with parasite Ag and cytokine production by draining LN cell cultures was determined 4 and 8 days after immunization. Immunization of mice with *T. cruzi* Ag-pulsed GM-MØ led 4 days later to significantly higher IFN-γ and IL-2 production by LN cells than observed after immunization only with the antigen or with *T. cruzi* Ag-pulsed M-MØ (Fig. 2A). Unstimulated cultures derived from GM-MØ immunized mice also showed higher IFN-γ production than those derived from M-MØ immunized mice (data not shown). IL-10 levels were reduced in LN from both Ag-pulsed GM-MØ- and M-MØ-immunized mice as compared with cultures from mice immunized only with *T. cruzi* Ag (Fig. 2A). IL-4 was below detection levels in all experimental situations. Cellular proliferation of LN cells, measured by [^{3}H]-TdR incorporation at 72 h of culture of Ag-stimulated cultures, was of similar magni-

Table 2
IL-12 production by M-MØ or GM-MØ infected with *T. cruzi*

Cells[a]	Stimulus	IL-12 (ng/ml)		
		Medium	IFN-γ (100 U/ml)[b]	IL-4 (10 ng/ml)[b]
M-MØ	–	<0.3[c]	<0.3	<0.3
	SaC	0.5 ± 0.1	<0.3	<0.3
	Live *T. cruzi*	1.6 ± 0.3	0.8 ± 0.2	<0.3
GM-MØ	–	7.7 ± 1.5	7.5 ± 1.5	1.0 ± 0.2
	SaC	20.1 ± 4.0*	24.1 ± 4.8*	3.7 ± 0.7**
	Live *T. cruzi*	27.5 ± 5.5*	26.8 ± 5.4*	4.3 ± 0.9**

[a] As described in Table 1. Representative of three experiments.

[b] As described in Table 1.

[c] Below minimal detection level. *$P < 0.05$ compared with identically stimulated and treated M-MØ. **$P < 0.05$ compared with GM-MØ without IL-4-stimulation.

Table 3
IL-12 production by M-MØ or GM-MØ pulsed with Tc-Ag

		[Tc-Ag] per 10^6 macrophages (μg)				
		2	10	50	250	500
M-MØ[a]	<0.3[b]	ND	ND	<0.3	<0.3	<0.3
GM-MØ	3.2 ± 0.6	2.6 ± 0.5	3.6 ± 0.7	5.0 ± 1.0	11.1 ± 3.2	13.5 ± 3.7

[a] Macrophages were cultured in vitro in medium containing different concentrations of Tc-Ag. 24 h later, all supernatants were harvested and IL-12 levels were determined by ELISA.
[b] IL-12 (p40) levels expressed in ng/ml. Means of duplicates determinations ± S.D.

tude in mice immunized with Ag-pulsed GM-MØ (86 616 ± 1552 cpm), in mice immunized with Ag-pulsed M-MØ (61 472 ± 11 641 cpm) and in mice immunized only with parasite Ag (66 192 ± 3875 cpm).

By day 8 after immunization, IFN-γ and IL-2 levels in LN cultures from Ag-pulsed-GM-MØ-immunized mice had fallen to 12.1 and 4.6 ng/ml, respectively, and were not significantly different from those observed for mice immunized with Ag-pulsed M-MØ, although Ag-specific proliferative responses were higher in the former group of mice (data not shown).

Taken together, these results indicate that immunization with Ag-pulsed-GM-MØ stimulates a stronger transient IFN-γ and IL-2 production in the draining LN than immunization with Ag-pulsed M-MØ.

3.5. Immunization with T. cruzi-Ag-pulsed GM-MØ or M-MØ followed by infection with live parasites

We next verified whether the higher stimulus for IFN-γ production by LN provided by immunization with *T. cruzi*-Ag-pulsed GM-MØ, as compared with pulsed M-MØ, would hold in a situation of subsequent infection with live parasites. Mice were immunized with *T. cruzi*-Ag pulsed GM-MØ or M-MØ and infected 24 h later with live *T. cruzi*. As shown in Fig. 2B, both immunization procedures resulted in enhanced Ag-stimulated IFN-γ production by lymph nodes 4 days after infection when compared with mice injected only with *T. cruzi*-Ag prior to infection. In the absence of exogenous stimuli added to the cultures (maintained only in culture medium), we also observed increased IFN-γ production in infected LN cultures from both GM-MØ and M-MØ immunized mice (range 20.1–34.9 vs 3.1 ng/ml in mice immunized only with Ag). No differences in IL-2 or IL-10 production levels were observed among the experimental groups.

In order to evaluate whether the higher earlier production of IFN-γ observed in *T. cruzi*-Ag pulsed GM-MØ or M-MØ immunized mice would modify the course of a subsequent infection in susceptible BALB/c mice, parasitemia and mortality levels were determined in these mice. No significant differences in parasitemia levels or in mortality levels were observed among the groups of mice that were immunized with *T. cruzi* Ag-pulsed macrophages or parasite Ag alone, or that were only infected (data not shown).

4. Discussion

The results reported in this paper show that GM-CSF and M-CSF stimulate bone-marrow macrophages to differentiate to functionally distinct populations regarding their ability to control in vitro *T. cruzi* infection in the presence of IFN-γ and to synthesize nitric

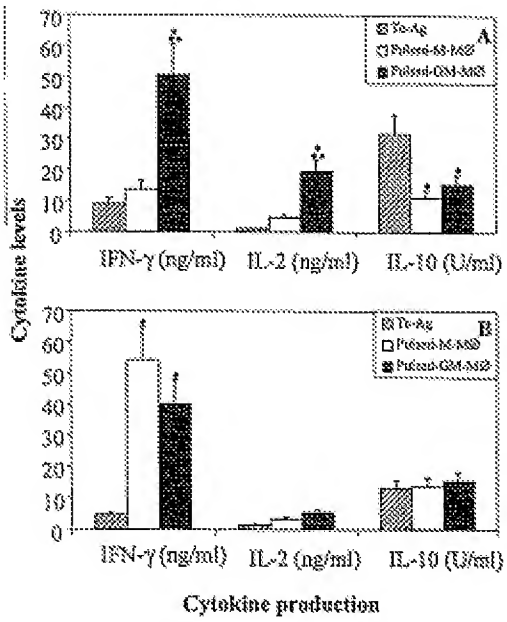

Fig. 2. Cytokine production by LN cell cultures from mice immunized with *T. cruzi*-Ag-pulsed GM-MØ or M-MØ (A) or immunized with pulsed-macrophages and infected 24 h later by the same route with 1000 live *T. cruzi* blood forms (B). Groups of five mice were immunized with 6×10^6 macrophages pulsed with Tc-Ag. A control group of mice was immunized only with Tc-Ag. LN cultures were established 4 days after immunization (A) or 4 days after infection (5 days after immunization, B) and stimulated with Tc-Ag. Supernatants were collected at 24 h for IL-2 and at 72 h for IFN-γ and IL-10 quantification. Means ± S.E.M. $^*P < 0.05$ compared with Tc-Ag group; $^{**}P < 0.05$ GM-MØ vs M-MØ immunized groups. Representative of two experiments.

oxide and IL-12. Both GM-MØ and M-MØ were susceptible to infection and growth of the parasites and both were activated by IFN-γ to a parasiticidal effect. However, IFN-γ-activated GM-MØ were more effective than IFN-γ-activated M-MØ at controlling intracellular parasitism. The enhanced parasiticidal effect correlated with the higher levels of NO produced by the former cell population. It has been shown earlier that mouse peritoneal MØ treated with IFN-γ and GM-CSF have increased production of NO and parasiticidal effect [5]. Unlike earlier results obtained by treating mouse peritoneal MØ with GM-CSF [5,6], we were unable to observe a trypanocidal or trypanostatic effect by GM-CSF alone on bone-marrow macrophages cultured in the presence of this cytokine. This discrepancy may reflect the different responsiveness between bone-marrow derived and peritoneal macrophages populations when exposed to the cytokine.

An interesting feature that distinguishes GM-MØ and M-MØ populations is the ability of GM-MØ to synthesize high levels of IL-12, when infected in vitro with live *T. cruzi* trypomastigotes or when stimulated with disrupted parasites A subclass of dendritic cells has been identified that, in the mouse, carries the CD8α marker, produces IL-12 and directs Th cell differentiation towards Th1 [28]. However, the phenotypic analysis of GM-MØ and M-MØ cultures on the day 10 of culture showed low and similar percentages (2.5 and 4.6%, respectively) of $CD11c^+/CD8\alpha^+$ cells. Thus, the higher production of IL-12 by GM-MØ cannot be ascribed to a higher frequency of contaminant dendritic cells of the lymphoid-type ($CD11c^+/CD8\alpha^+$ cells) in GM-CSF derived cultures as compared with M-CSF-derived cultures. Moreover, GM-MØ cultures had also a lower percentage of $CD11c^+/CD8\alpha^-$ dendritic cells. IL-12 is known as a cytokine that stimulates IFN-γ production by NK and T cells [27]. The importance of IL-12 in the control of *T. cruzi* parasitism by innate immunity was confirmed by experiments that showed the aggravation of the infection in mice devoid of T and B cells (RAG/KO mice) submitted to anti-IL-12 mAb treatment [10]. Treatment with anti-IL-12 antibody also rendered immunocompetent mice more susceptible to *T. cruzi* infection and resulted in diminished production of IFN-γ and NO by spleen cells [10,11]. The question arose whether GM-MØ stimulated with parasite antigen would be able to potentiate in vivo the immune response leading to protection against *T. cruzi* infection. An existing report describing increased resistance of BALB/c mice to *L. major* infection, conferred by immunization with *Leishmania* Ag primed GM-MØ [12], shows that this procedure enhances draining LN production of IFN-γ [12]. A recent report by the same authors correlates these results to a transient increase in IL-12 production by LN in those mice [13].

Our results show that immunization with *T. cruzi* Ag-pulsed GM-MØ, but not with pulsed M-MØ or with antigen alone, resulted in a transient elevation of IFN-γ and IL-2 production by in vitro Ag-stimulated LN cells. However, stimulation of IFN-γ production was observed in both GM-MØ- and M-MØ-immunized groups of mice when they were challenged with live *T. cruzi* by the same route (s.c.) as the one used for immunization. This result suggests that the cellular microenvironment of the LN challenged with live parasites provided additional stimulation to M-MØ that resulted in enhanced stimulation of IFN-γ synthesis by lymphocytes. It has been shown that peritoneal macrophages infected with *T. cruzi* produce GM-CSF [5]. Moreover, exposure of M-MØ to GM-CSF during 24 h was enough to stimulate the synthesis of a T cell stimulatory factor (TSF) that stimulates IFN-γ production [15]. Furthermore, GM-CSF increases the production of IL-12 induced by *S. aureus* or LPS in macrophage cultures [27]. In this context, it is possible that GM-CSF produced by *T. cruzi* infected LN macrophages could stimulate bystander injected M-MØ to secrete IL-12 leading to increased IFN-γ synthesis.

Unlike the results reported for the *Leishmania major* model of infection [12], we did not observe increased resistance to *T. cruzi* in the mice immunized with parasite Ag-primed macrophage populations. This result probably reflects the systemic nature of *T. cruzi* infection with parasites rapidly disseminating to other organs by the blood stream as opposed to *Leishmania* infection that remains for a longer time confined to the skin and draining LN.

In conclusion, we have shown that GM-CSF and M-CSF stimulate bone-marrow macrophages to differentiate to functionally distinct populations regarding their ability to control in vitro *T. cruzi* infection, to synthesize IL-12 and nitric oxide and to prime the immune response to parasite antigen. The results reinforce the role of GM-CSF as a cytokine that, acting on macrophages, can participate in the innate control of intracellular parasitism and enhance innate immunity.

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EXHIBIT 6

Granulocyte-Macrophage Colony-Stimulating Factor (CSF) and Macrophage CSF-Dependent Macrophage Phenotypes Display Differences in Cytokine Profiles and Transcription Factor Activities: Implications for CSF Blockade in Inflammation[1]

Andrew J. Fleetwood,[2]* Toby Lawrence,† John A. Hamilton,* and Andrew D. Cook*

GM-CSF and M-CSF (CSF-1) can enhance macrophage lineage numbers as well as modulate their differentiation and function. Of recent potential significance for the therapy of inflammatory/autoimmune diseases, their blockade in relevant animal models leads to a reduction in disease activity. What the critical actions are of these CSFs on macrophages during inflammatory reactions are unknown. To address this issue, adherent macrophages (GM-BMM and BMM) were first derived from murine bone marrow precursors by GM-CSF and M-CSF, respectively, and stimulated in vitro with LPS to measure secreted cytokine production, as well as NF-κB and AP-1 activities. GM-BMM preferentially produced TNF-α, IL-6, IL-12p70, and IL-23 whereas, conversely, BMM generated more IL-10 and CCL2; strikingly the latter population could not produce detectable IL-12p70 and IL-23. Following LPS stimulation, GM-BMM displayed rapid IκBα degradation, RelA nuclear translocation, and NF-κB DNA binding relative to BMM, as well as a faster and enhanced AP-1 activation. Each macrophage population was also pretreated with the other CSF before LPS stimulation and found to adopt the phenotype of the other population to some extent as judged by cytokine production and NF-κB activity. Thus, GM-CSF and M-CSF demonstrate, at the level of macrophage cytokine production, different and even competing responses with implications for their respective roles in inflammation, including a possible dampening or suppressive role for M-CSF in certain circumstances. *The Journal of Immunology,* **2007, 178: 5245–5252.**

The phenotypic diversity of macrophage lineage populations leading to divergent functions, for example at sites of inflammation or immunity, is becoming apparent (1). These cells are able, through the vast array of mediators that they are capable of producing, to control both the progression and resolution of inflammatory lesions (2, 3). Attempts have been made to clarify macrophage phenotypes (subsets) and activation states resulting from exposure to the various stimuli. For example, polarization into the so-called M1 and M2 states has been proposed (2, 4) as has alternate activation states characterized by a classical (proinflammatory) or a nonclassical phenotype (5).

Two cytokines, which appear to be important in controlling the numbers and function of macrophage lineage populations in inflammatory conditions, are GM-CSF and M-CSF; also known as CSF-1 (6–8). These CSFs are also critical to the proper maintenance of steady-state macrophage development, although with different roles. Studies using mice deficient in functional M-CSF (*op/op* mice) revealed that M-CSF plays a role in the steady-state development of a number of macrophage populations (9, 10). For mice deficient in GM-CSF, the major pathology is alveolar proteinosis (11). GM-CSF has been shown to be essential for proper alveolar macrophage maturation (12).

The CSFs are prosurvival/mitogenic factors for macrophage lineage populations; they are also able to "prime" or "activate" macrophages as well as induce their differentiation (7, 13). In vitro studies of CSF action on monocytes/macrophages have shown that they can modulate the expression of common and divergent sets of products (6, 14). GM-CSF is often used in the presence of a costimulus such as IL-4 to generate dendritic cell (DC)[3] populations (15) whereas M-CSF is critical for the control of osteoclast development (10).

There have been studies in the mouse comparing the actions of these CSFs by generating cell populations via proliferation and differentiation of bone marrow precursors. The GM-CSF-dependent cells derived in this way differ from their M-CSF-derived counterparts in a number of ways (16–19). There is also evidence that when both CSFs are added to the same monocyte/macrophage population they can compete, leading to a suppression of the cellular response to the other CSF (20, 21). Recently, proinflammatory type-1 and anti-inflammatory type-2 human monocyte-derived macrophage subsets (termed Mϕ-1 and Mϕ-2) have been generated in the presence of GM-CSF and M-CSF, respectively (1, 22). However, neither the molecular events contributing to the

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[3] Abbreviations used in this paper: DC, dendritic cell; BMM, M-CSF-dependent murine bone marrow-derived macrophage; GM-BMM, GM-CSF-dependent murine bone marrow-derived macrophage; Mϕ-1, proinflammatory type-1 human monocyte-derived subset; Mϕ-2, anti-inflammatory type-2 human monocyte-derived macrophage subset; Q-PCR, quantitative PCR.

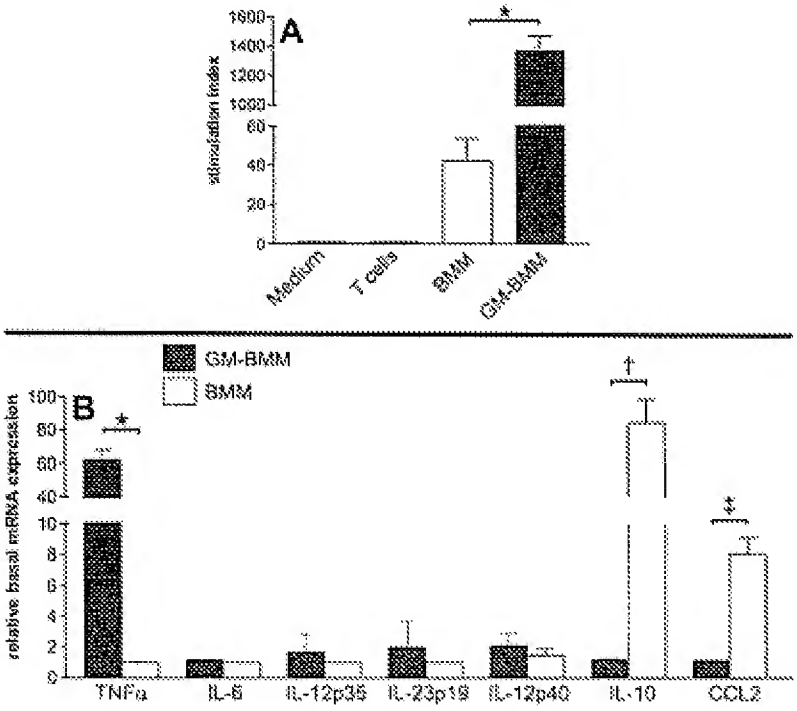

FIGURE 1. Differential stimulatory activity and basal cytokine mRNA expression of GM-BMM and BMM. *A*, The stimulatory activity of GM-BMM and BMM was measured in an allogeneic MLR. GM-BMM and BMM cells were cultured in triplicate with allogeneic splenic T cells (2×10^5 cells/well) at a ratio of 1:10 for 96 h at 37°C in 5% CO_2. Cultures were pulsed with [^{3}H]TdR for the last 16 h to measure DNA synthesis. Control cultures contained medium or T cells alone. Background counts per minute for medium alone was 108 ± 16. The results represent the mean ± SEM of values from three independent experiments. *, $p < 0.01$, for comparison of the stimulation index of GM-BMM vs BMM. *B*, Basal cytokine mRNA expression in GM-BMM and BMM as measured by Q-PCR. Levels of TNF-α, IL-6, IL-12p35, IL-23p19, IL-12p40, IL-10, and CCL2 mRNA were normalized to an endogenous reference (18S) and calibrated to the lowest expression level for each cytokine. The results represent the mean ± SEM of values from four independent experiments. *, $p < 0.01$, GM-BMM vs BMM for TNF-α; †, $p < 0.01$, BMM vs GM-BMM for IL-10; ‡, $p < 0.05$, BMM vs GM-BMM for CCL2.

different properties of these subsets nor the potential interplay of the respective CSFs in this system was examined.

In the current study, we generated from murine bone marrow precursors GM-CSF- and M-CSF-dependent macrophage populations (GM-BMM and BMM, respectively) and studied, following LPS activation, both the cytokine expression and some of the molecular changes that might help to explain differences in such expression. We also included an analysis of the interplay between the CSFs on subsequent macrophage activation.

Materials and Methods

Mice

Female C57BL/6 mice (8–12 wk) were supplied by Monash University, Central Animal Services (Parkville, Victoria, Australia).

Reagents

Reagents were as follows: recombinant murine GM-CSF (PeproTech) and recombinant human M-CSF (Chiron); Abs against RelA, c-Fos, JunB, c-Jun, JunD, β-actin, and β-tubulin (Cell Signaling Technology); p50 Ab (eBioscience); and LPS (*Escherichia coli* serotype 0127:B8; Sigma-Aldrich).

Preparation of bone marrow-derived macrophages

Bone marrow-derived macrophages grown in M-CSF or GM-CSF were generated as previously described (23). On day 7 adherent GM-BMM and BMM (5×10^5 cells/ml) were stimulated with LPS (100 ng/ml) for the indicated time points. For "priming" experiments, BMM at day 7 were stimulated for 16 h with GM-CSF (+GM-CSF; 1000 U/ml GM-CSF and 1000 U/ml M-CSF) or without GM-CSF (−GM-CSF; 1000 U/ml M-CSF). Similarly, GM-BMM at day 7 were stimulated for 16 h with M-CSF (+M-CSF; 1000 U/ml M-CSF and 1000 U/ml GM-CSF) or without M-CSF (−M-CSF; 1000 U/ml GM-CSF). Following pretreatment, BMM and GM-BMM were stimulated with fresh medium containing LPS (100 ng/ml).

Cytokine ELISAs

TNF-α, IL-6, IL-10, IL-12p70, IL-12p40, and CCL2 (OptEIA ELISA kits; BD Pharmingen) and IL-23p40/p19 (eBioscience) content were measured in culture supernatants by ELISA.

Mixed Leukocyte Reaction

The MLR was performed as previously described (24). Briefly, purified T cells (2×10^5 cells/well) were cultured with BMM and GM-BMM for 96 h at 37°C in 5% CO_2 such that the final E:T ratio was 1:10. Control cultures contained medium or T cells alone. Before harvesting (16 h), cells were pulsed with 1 μCi of [^{3}H]TdR (Amersham Biosciences) and DNA synthesis was measured by [^{3}H]TdR incorporation using a Beckman beta scintillation counter (Beckman Instruments). Results are expressed as the stimulation index above medium alone.

Quantitative PCR (Q-PCR) analysis of gene expression

Quantitative-PCR (Q-PCR) was performed as before (23). Predeveloped TaqMan probe/primers for TNF-α, IL-6, IL-12p35, IL-12p40, IL-23p19, IL-10, and CCL2 (Applied Biosystems) were used to calculate the threshold cycle numbers that were transformed using the cycle threshold and relative value method as described by the manufacturer and expressed relative to 18S ribosomal RNA. Results are expressed as relative gene expression for each target gene.

Protein extraction and Western blot analysis

Whole cell extracts were lysed directly and Western blotting was performed by standard techniques (25). Briefly, cells (3×10^6) were lysed in tissue culture dishes with Nonidet P-40 lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate, 0.1 mM sodium molybdate, 10 mM NaF, 5 mM β-glycerophosphate, and Complete protease inhibitors) for 30 min on ice. Lysates were clarified by centrifugation at 13,000 × *g* for 10 min at 4°C and protein concentrations were measured with a Bio-Rad assay kit. All extracts were stored at −80°C until use. Proteins were separated on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane followed by immunoblotting for specific Abs as per the manufacturer's instructions. Densitometry was performed using Kodak EDAS 1D image analysis software.

EMSA and supershift analysis

Generation of nuclear lysates and EMSAs were conducted as before (26). Briefly, cells (8×10^6) were lysed in hypotonic lysis buffer (0.0125% Nonidet P-40, 5 mM HEPES (pH 7.8), 10 mM KCl, and 1.5 mM $MgCl_2$) and nuclei were harvested by centrifugation (13,000 × *g* for 10 min). Nuclear pellets were incubated in hypertonic extraction buffer (0.025% Nonidet P-40, 5 mM HEPES (pH 7.9), 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, and 0.2 mM EDTA) for 15 min at 4°C. Nuclear extracts (5 μg) were incubated with labeled probe (50,000 cpm), and resolved on a gel in 0.5× Tris borate-EDTA at 200 V for 20–30 min. Complexes were visualized using Super RX (Fuji Film). For supershift experiments, nuclear extracts were pretreated with 1 μl of a particular Ab and DNA binding complexes were resolved on a nondenaturing 5% (w/v) polyacrylamide gel

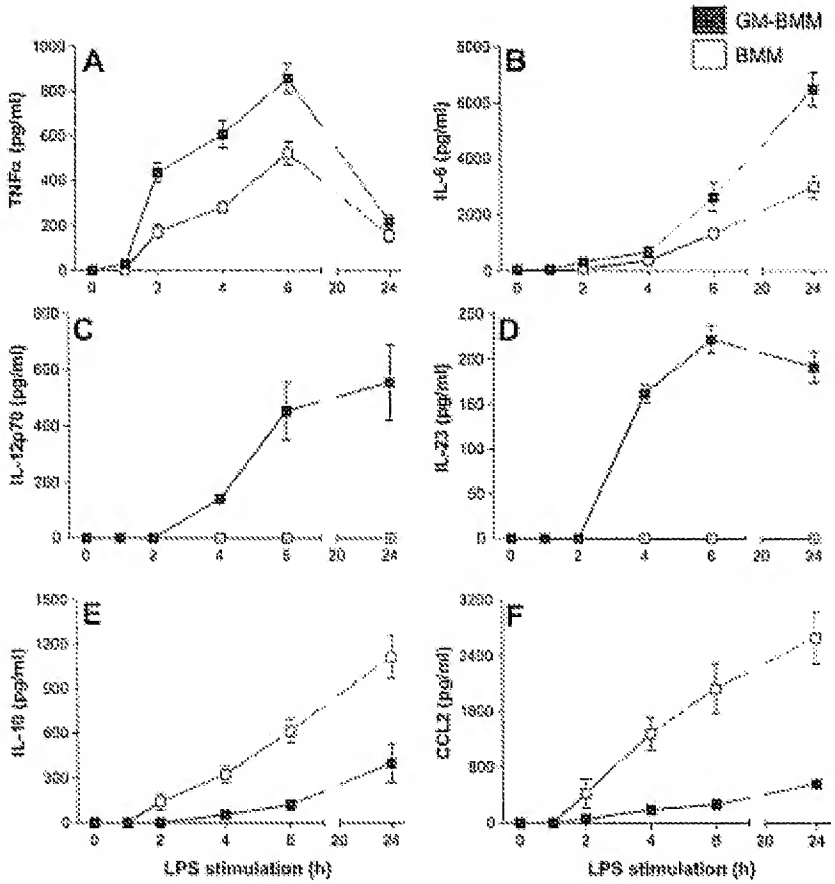

FIGURE 2. Differential cytokine production from GM-BMM and BMM following LPS stimulation. TNF-α (*A*), IL-6 (*B*), IL-12p70 (*C*), IL-23 (*D*), IL-10 (*E*), and CCL2 (*F*) levels from GM-BMM and BMM following LPS (100 ng/ml) stimulation are shown. The results represent the mean ± SEM of values from four independent experiments. Where not visible, error bars are smaller than the symbol. $p < 0.01$, GM-BMM vs BMM for TNF-α, IL-6, IL-12p70, and IL-23 (*A–D*); $p < 0.05$, BMM vs GM-BMM for IL-10 and CCL2 (*E* and *F*).

in 0.1% Tris borate-EDTA. Gels were dried onto 3-mm paper (Whatman) and complexes were visualized as described above.

Statistics

Data are given as mean values ± SEM. Statistical significance was evaluated over the 24-h LPS stimulation period by two-way ANOVA and $p < 0.05$ was considered statistically significant.

Results

Phenotyping GM-BMM and BMM

Murine bone marrow cells were cultured in vitro for 7 days in the presence of GM-CSF or M-CSF and the adherent cells (GM-BMM and BMM, respectively) were collected. We have shown elsewhere (23) that both populations express common macrophage markers (Mac-1 (CD11b), F4/80, and c-Fms (M-CSFR)), whereas only GM-BMM express CD11c, an integrin often used in murine systems to identify DC lineage cells but also found on certain macrophage populations (e.g., alveolar macrophages (12)). We have also recently demonstrated that both cell types can be quantitatively converted to osteoclast lineage cells (23). In the current study, we demonstrate that GM-BMM cells, relative to BMM cells, are potent stimulators of T cell proliferation in a MLR. As can be seen in Fig. 1*A*, GM-BMM cells were ~30-fold more potent than BMM cells at stimulating allogeneic T cells in a MLR. We refer here to the GM-CSF-derived adherent cells as GM-BMM although they are functionally sometimes referred to as DCs (27).

Cytokine production from GM-BMM and BMM following LPS stimulation

Before LPS stimulation, GM-BMM and BMM cells were unable to secrete detectable TNF-α, IL-6, IL-12p70, IL-23, IL-10, or CCL2 (data not shown), although by Q-PCR we found the basal mRNA expression levels of TNF-α, IL-10, and CCL2 were significantly different (Fig. 1*B*). The relative basal mRNA levels of TNF-α ($p < 0.01$; Fig. 1*B*) were enhanced from GM-BMM compared with BMM, whereas the relative basal mRNA levels of the anti-inflammatory cytokine IL-10 ($p < 0.01$; Fig. 1*B*) and the chemokine CCL2 (MCP-1) ($p < 0.05$; Fig. 1*B*) were enhanced in BMM compared with GM-BMM. There were no significant differences in the basal mRNA levels for IL-6 or for the IL-12 and IL-23 subunits (IL-12p35, IL-23p19, and IL-12/IL-23 shared subunit p40) between the two macrophage populations. We then went on to determine the cell surface expression of TLR4 and TLR2. As has previously been demonstrated (28), both cell types were uniformly positive for TLR2 and TLR4 and their mean fluorescence intensities were similar (data not shown).

Following LPS stimulation, GM-BMM produced significantly more TNF-α ($p < 0.01$) and IL-6 ($p < 0.01$) compared with BMM (Fig. 2, *A* and *B*, respectively). Both IL-12p70 and IL-23 are composed of two subunits, a common p40 subunit and either a p35 or a p19 subunit, respectively (1). GM-BMM secreted both IL-12p70 and IL-23 following LPS stimulation whereas BMM failed to do so over the 24-h period examined ($p < 0.01$; Fig. 2, *C* and *D*, respectively). LPS-stimulated BMM produced IL-12p40 but at levels lower than for GM-BMM (data not shown). In contrast to the above cytokines and consistent with the basal mRNA expression pattern (Fig. 1*B*), LPS-stimulated BMM secreted more IL-10 and CCL2 ($p < 0.05$; Fig. 2, *E* and *F*, respectively). The divergent production of the above mediators by GM-BMM and BMM correlated with significantly different LPS-induced mRNA levels of TNF-α, IL-12p35, IL-23p19, IL-12p40, IL-10, and CCL2 (data not shown).

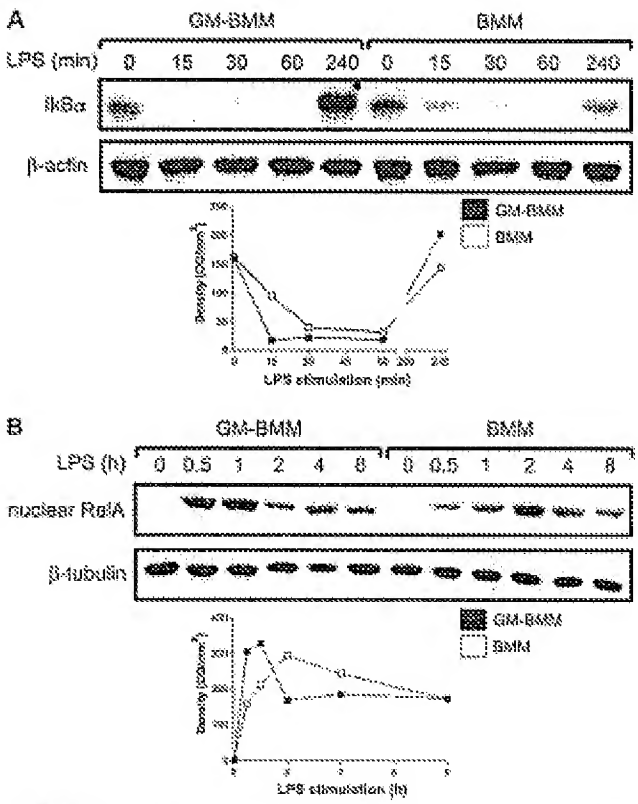

FIGURE 3. IκBα degradation and RelA nuclear translocation in GM-BMM and BMM following LPS stimulation. *A*, IκBα degradation was analyzed by Western blotting of whole cell lysates. *B*, Nuclear translocation of RelA was analyzed by Western blotting. β-Actin and β-tubulin levels were used as loading controls. Densitometric analysis was performed using Quantity One software and data are expressed as density (OD/mm^2) relative to that for the β-actin/β-tubulin. Representatives of three independent experiments are shown.

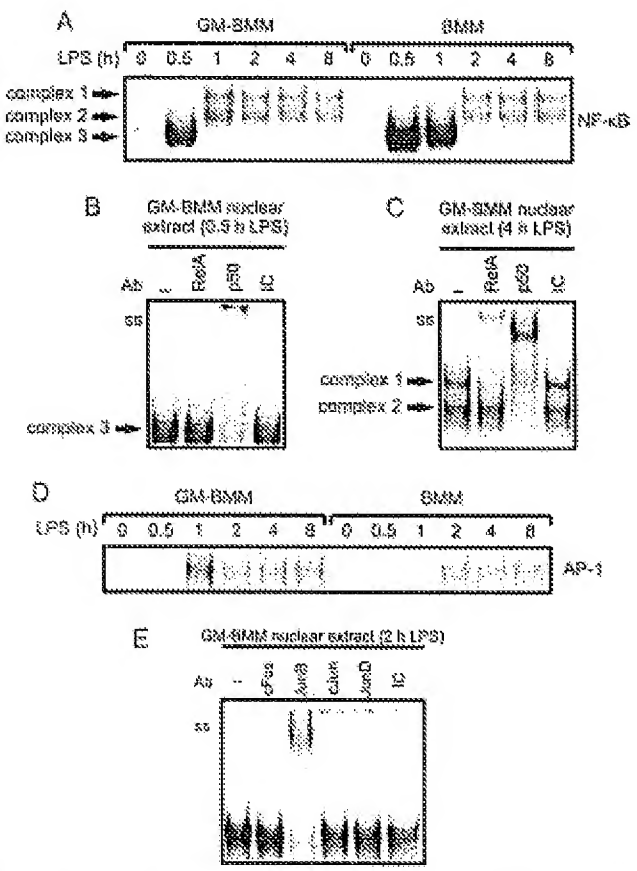

FIGURE 4. NF-κB and AP-1 DNA binding in GM-BMM and BMM following LPS stimulation. *A*, NF-κB DNA binding activity was analyzed by EMSA. *B* and *C*, Supershift analysis of NF-κB DNA binding complexes with Abs recognizing indicated NF-κB proteins and isotype control (IC) Ab. *D*, AP-1 DNA binding activity was analyzed by EMSA. *E*, Supershift analysis of the AP-1 DNA binding complex with Abs recognizing the indicated AP-1 proteins and the isotype control (IC) Ab. Representatives of three independent experiments are shown.

NF-κB and AP-1 DNA binding activity in GM-BMM and BMM following LPS stimulation

NF-κB activity. Because NF-κB regulates the expression of multiple genes important in immunologic and inflammatory responses (29), we reasoned that there may be differential NF-κB activation.

As can be seen in Fig. 3*A*, the rate of IκBα degradation was different between GM-BMM and BMM in response to LPS. IκBα was completely degraded in GM-BMM within 15 min of LPS stimulation; IκBα could still be detected in BMM at this time point. In both GM-BMM and BMM, IκBα levels were restored after 4 h of LPS stimulation. We next examined translocation of the RelA subunit into the nucleus. Western blot analysis demonstrated that maximal GM-BMM RelA nuclear levels peaked at ~0.5–1 h after LPS stimulation (Fig. 3*B*) and appeared to be slightly greater than those for BMM; for BMM, maximal nuclear RelA levels were not reached until ~2 h after LPS stimulation (Fig. 3*B*).

To assess the NF-κB DNA binding activity, EMSAs were conducted on nuclear extracts. Before LPS stimulation, NF-κB DNA binding complexes could not be detected in either GM-BMM or BMM (Fig. 4*A*). However, following LPS addition three distinct NF-κB DNA binding complexes (designated complexes 1, 2, and 3) were formed in both cell populations. Interestingly, the kinetics of complex formation differed. Both GM-BMM and BMM rapidly formed complex 3 within 30 min of LPS stimulation. However, by 1 h of treatment in GM-BMM complex 3 had disappeared and complexes 1 and 2 had formed, whereas in BMM complex 3 was still present while the formation of complexes 1 and 2 was delayed until 2 h after LPS activation. Both complexes 1 and 2 were maintained over the duration of the 8-h stimulation period examined in both cell populations.

To assess the composition of the three complexes, supershift analysis of GM-BMM treated with LPS for 30 min and 4 h was conducted. For the 30-min LPS treatment anti-p50 Ab completely displaced complex 3 (supershift), while anti-RelA failed to supershift it (Fig. 4*B*). This observation is consistent with complex 3 containing p50, most likely as a homodimer (30). Supershift analysis of GM-BMM following 4 h LPS stimulation was used to help reveal the composition of complexes 1 and 2. Fig. 4*C* demonstrates that the anti-RelA Ab displaced complex 1 (supershift) whereas anti-p50 Ab displaced both complex 1 and complex 2 (supershift). Supershift analyses of the corresponding time points for BMM lysates gave similar results (data not shown).

In summary, following LPS treatment GM-BMM, compared with BMM, displayed more rapid IκBα degradation, RelA translocation and formation of a RelA-containing DNA binding complex. It is possible that these differences may relate to the heightened expression in GM-BMM of TNF-α, IL-12p70, and IL-23 (Fig. 2), for example, the genes of which have been shown to be regulated by NF-κB activity (29).

AP-1 activity. Cooperation between NF-κB and AP-1 is known to be important for the activation of certain cytokine promoters (31).

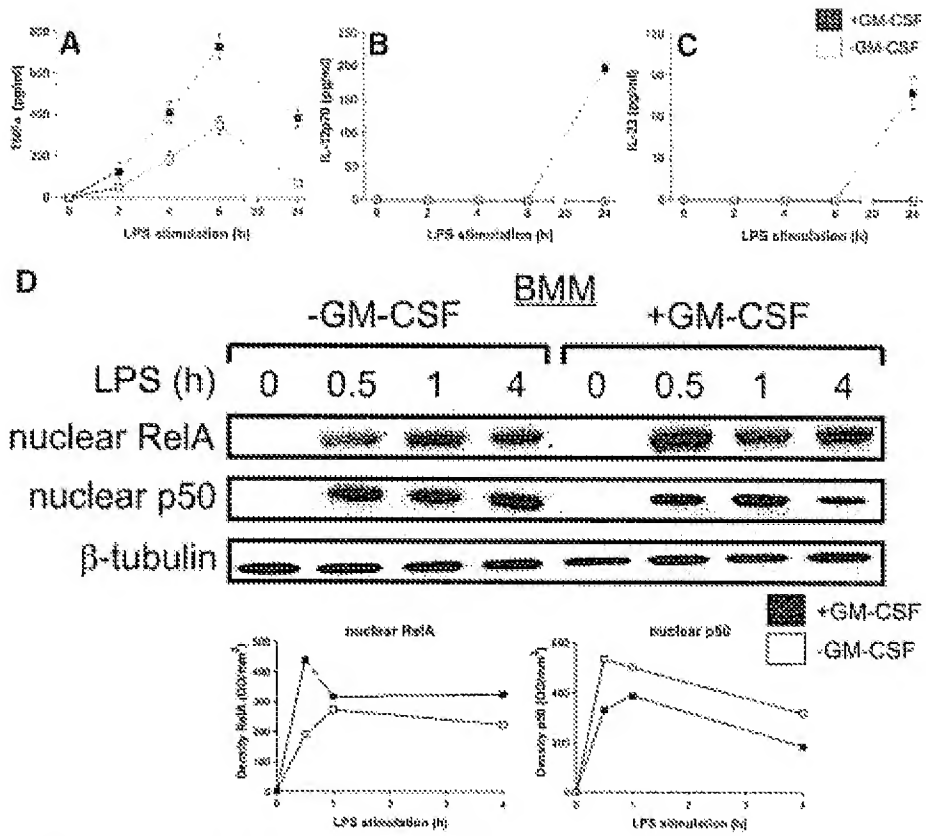

FIGURE 5. Cytokine production and nuclear localization of RelA and p50 from LPS-stimulated BMM following pretreatment with GM-CSF. Day 7 BMM cells were pretreated with or without GM-CSF (1000 U/ml) in the presence of M-CSF for 16 h and then stimulated with LPS. *A–C*, TNF-α (*A*), IL-12p70 (*B*), and IL-23 (*C*) production was measured. The results represent the mean ± SEM of cytokine values from four independent experiments. Where not visible, error bars are smaller than the symbol. $p < 0.05$, +GM-CSF vs −GM-CSF for TNF-α (*A*); $p < 0.01$, +GM-CSF vs −GM-CSF for IL-12p70 (*B*) and IL-23 (*C*). *D*, Nuclear localization of RelA and p50 was measured by Western blotting in lysates from BMM, pretreated with or without GM-CSF. Densitometric analysis was performed using Quantity One software and data are expressed as density (OD/mm²) relative to that for β-tubulin. Representatives of three independent experiments are shown.

As for NF-κB, before LPS stimulation no AP-1 binding could be detected in either GM-BMM or BMM (Fig. 4*D*). Following LPS addition, a single DNA binding complex was evident for both cell types with the maximal activity being higher in GM-BMM. Similar to the NF-κB DNA binding kinetics (Fig. 4*A*), those for AP-1 DNA binding were also different. AP-1 DNA binding was rapidly induced within 1 h after LPS treatment in GM-BMM; the formation of AP-1 DNA binding was delayed in BMM until around 2 h after LPS addition. In both GM-BMM and BMM, AP-1 DNA binding was maintained over the 8-h LPS stimulation period examined.

Supershift analysis of GM-BMM following 2 h of LPS treatment was used to assess the composition of the DNA binding complex. The anti-JunB Ab completely displaced the complex (supershift), while anti-c-Fos, anti-c-Jun, and anti-JunD failed to supershift it (Fig. 4*E*). This observation is consistent with the AP-1 DNA binding complex containing JunB, which is known to form transcriptionally active homodimers (32). Supershift analysis of the corresponding time point for BMM lysates gave similar results (data not shown).

Effect of the coaddition of GM-CSF and M-CSF on cytokine production following LPS stimulation

Particularly at sites of inflammation (33, 34) it is likely that macrophage populations in vivo will be exposed to both CSFs. We next addressed the question as to whether the respective secretory phenotypes of GM-BMM and BMM could be converted to that of the other, at least to some extent.

BMM pretreated with GM-CSF. To assess the effect that GM-CSF has on BMM cytokine production, BMM cells were cultured for an additional 16 h with or without GM-CSF (M-CSF was also added to both treatment groups during this period). Following GM-CSF pretreatment, BMM cells were not significantly altered in terms of their cell surface Ag expression, at least as judged by CD11c, TLR2, and TLR4 levels (data not shown). However, following the GM-CSF pretreatment of BMM, basal mRNA levels of TNF-α, IL-23p19, and IL-12p40 were increased while IL-10 mRNA levels were decreased (data not shown). Consistent with the altered basal mRNA expression, following LPS stimulation GM-CSF-treated BMM cells produced increased amounts of TNF-α ($p < 0.05$; Fig. 5*A*) and were now capable of producing IL-12p70 (Fig. 5*B*) and IL-23 (Fig. 5*C*), albeit at lower levels and with delayed kinetics compared with that found for GM-BMM (Fig. 2, *C* and *D*). BMM cells pretreated with GM-CSF were also capable of significantly greater IL-12p40 production after LPS activation (data not shown). Interestingly, the levels of IL-10 and CCL2, the mediators whose levels were higher for BMM compared with GM-BMM following LPS stimulation (Fig. 2, *E* and *F*), were not altered by GM-CSF pretreatment (data not shown).

Western blot analysis demonstrated that GM-CSF pretreatment of BMM enhanced the nuclear localization of RelA while decreasing that of p50 (Fig. 5*D*). By EMSA, in BMM pretreated with GM-CSF there was an increased formation of complex 1 compared with control BMM grown solely in M-CSF at 1 and 4 h after LPS stimulation (data not shown). Supershift analysis, as

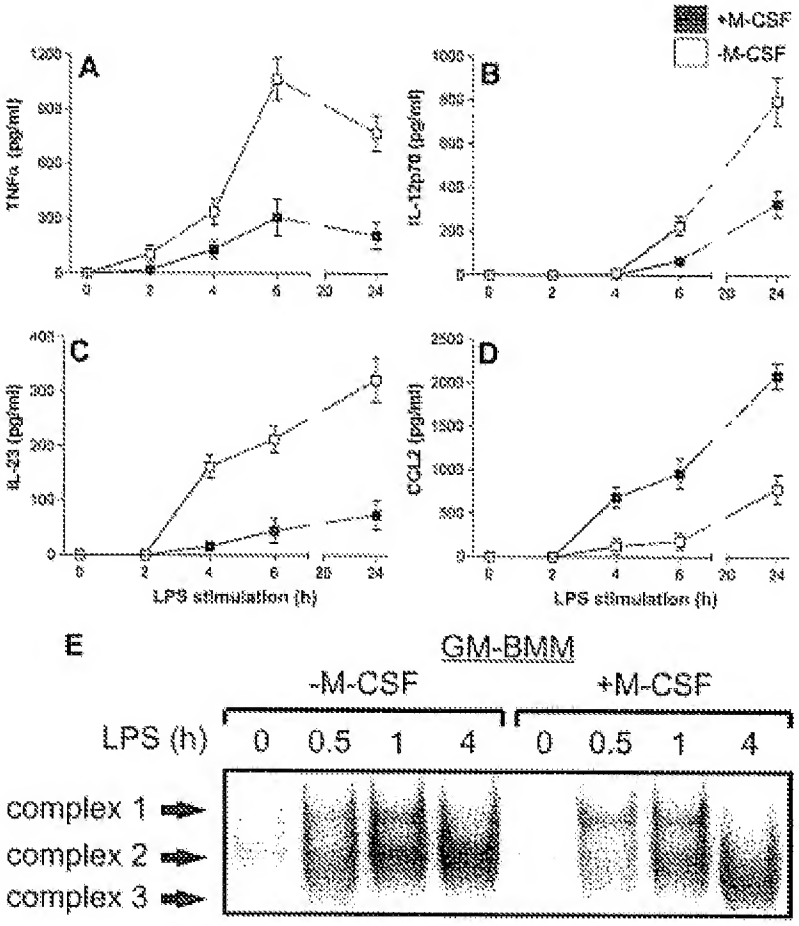

FIGURE 6. Modulation of cytokine production and NF-κB DNA binding activity from LPS-stimulated GM-BMM following pretreatment with M-CSF. Day 7 GM-BMM were treated with or without M-CSF (1000 U/ml) in the presence of GM-CSF for 16 h and then stimulated with LPS. *A–D*, TNF-α (*A*), IL-12p70 (*B*), IL-23 (*C*), and CCL2 (*D*) production was measured. The results represent the mean ± SEM of cytokine values from four independent experiments. Where not visible, error bars are smaller than the symbol. $p < 0.05$, −M-CSF vs +M-CSF for TNF-α (*A*), IL-12p70 (*B*), and IL-23 (*C*); $p < 0.05$, +M-CSF vs −M-CSF for CCL2 (*D*). *E*, NF-κB DNA binding activity was analyzed by EMSA using nuclear lysates from GM-BMM treated with or without M-CSF (1000 U/ml) for 16 h and then stimulated with LPS. Representatives of three independent experiments are shown.

in Fig. 4*C*, revealed that complex 1 contained transcriptionally active RelA. This increased formation of RelA-containing complexes following GM-CSF pretreatment correlated with the enhanced relative nuclear localization of RelA (Fig. 5*D*).

GM-BMM pretreated with M-CSF. Likewise, GM-BMM cells were cultured for an additional 16 h with or without M-CSF (GM-CSF was also added to both treatment groups during this period). Again, flow cytometry revealed no significant changes in CD11c, TLR2, or TLR4 levels following pretreatment with M-CSF (data not shown). However, following the pretreatment, mRNA levels of TNF-α, IL-23p19, and IL-12p40 were decreased while CCL2 mRNA levels were enhanced (data not shown). These altered basal mRNA levels correlated with the subsequent diminished LPS-induced production of TNF-α ($p < 0.05$; Fig. 6*A*) and IL-12p70 ($p < 0.05$; Fig. 6*B*) by GM-BMM cells pretreated with M-CSF; these cells also produced significantly less IL-23 ($p < 0.05$; Fig. 6*C*). The M-CSF pretreatment led to significantly greater production of CCL2 ($p < 0.05$; Fig. 6*D*) but once again there was no effect on the production of IL-10 (data not shown).

GM-BMM pretreated with M-CSF rapidly formed complexes 1 and 2 after LPS stimulation (Fig. 6*E*, +M-CSF), as did the control GM-BMM grown solely in GM-CSF (Fig. 6*E*, −M-CSF). However, by 4 h after LPS stimulation complex 3 was the major complex present in the GM-BMM cells pretreated with M-CSF, whereas complexes 1 and 2 were still present in the control GM-BMM cells grown solely in GM-CSF (Fig. 6*E*). Supershift studies of the two treatment groups following 4 h of LPS stimulation revealed that complex 1, formed in the control GM-BMM, contained RelA whereas complex 3, formed in GM-BMM pretreated with M-CSF, corresponded to the transcriptionally inactive p50 homodimer (see Fig. 4, *B* and *C*).

It was noted that the control GM-BMM cells that received the additional GM-CSF without M-CSF displayed altered NF-κB DNA binding kinetics. NF-κB DNA binding complexes 1 and 2 were seen 0.5 h after LPS stimulation in these GM-BMM cells (Fig. 6*E*, −M-CSF) whereas in day 7 GM-BMM, which last received GM-CSF on day 4 of culture, these complexes were not detected until 1 h after LPS stimulation (Fig. 4*A*). Similarly, the control BMM that received the additional M-CSF without GM-CSF formed NF-κB DNA binding complexes 1 and 2 at 1 h after LPS stimulation (data not shown), whereas we showed earlier that in day 7 BMM cells, which last received M-CSF on day 4 of culture, these complexes were not detected until 2 h after LPS stimulation (Fig. 4*A*). The addition of M-CSF to BMM before LPS activation has been shown to enhance the NF-κB activity compared with BMM "starved" of M-CSF (35).

In summary, it is possible that the above effects on NF-κB may relate to the altered expression of, for example, TNF-α, IL-12p70, and IL-23 from BMM and GM-BMM cells following GM-CSF or M-CSF pretreatment (Figs. 5 and 6).

Discussion

We showed above that LPS stimulation led to a very different pattern of cytokine production, with the GM-BMM cells preferentially producing TNF-α, IL-6, IL-12p70 and IL-23 and the BMM cells preferentially producing IL-10 and CCL2. What was particularly striking was the lack of production of IL-12p70 and IL-23 from the LPS-stimulated BMM. These findings are similar to what has been found recently when human monocytes

were differentiated by GM-CSF or M-CSF with the resultant macrophage populations termed Mϕ-1 and Mϕ-2, respectively (1, 32). In terms of their cytokine profile, GM-BMM resemble classically activated or M1-polarized macrophages that result in response to IFN-γ or microbial products (5), which is perhaps not surprising given that GM-CSF and IFN-γ have many of the same "priming" properties on monocytes and macrophages (36). As for M1 macrophages (4, 5), GM-BMM cells were weak producers of IL-10.

In contrast, following LPS stimulation BMM cells appear similar to the Mϕ-2 human monocyte-derived macrophages with an IL-12^{low}IL-10^{high} phenotype and with the capability of high CCL2 production (1, 22). It has been proposed that Mϕ-2 cells may play a role in both attracting, via chemokine activity, and then suppressing, via IL-10, adaptive immune cells (1, 22). In the mouse, ligation of a Fc receptor followed by TLR, CD40, or CD44 stimulation also gives rise to macrophages with an IL-12^{low}IL-10^{high} phenotype that are called type II-activated macrophages (5). BMM cells also seem similar to tumor-associated macrophages with a polarized M2 phenotype capable of high IL-10 production (4, 37). The contribution of endogenous IL-10 (38) to cytokine protein expression in BMM cells is currently being examined.

We also analyzed NF-κB and AP-1 activation. Following LPS stimulation, GM-BMM showed a faster IκBα degradation that correlated with the faster nuclear translocation of RelA and the formation of a NF-κB DNA binding complex containing RelA. This subunit can form homodimers or the classical RelA/p50 heterodimer, both of which are potent activators of gene expression (38). Even though we could not detect NF-κB activity in GM-BMM in the absence of LPS stimulation there were differences in basal cytokine mRNA expression from BMM, which might be due to differences in the levels of NF-κB/Rel family members; in this context, a study profiling gene expression in DCs, generated by culturing human monocytes in GM-CSF and IL-4, and in macrophages, generated by culturing them in M-CSF, showed that the expression of several NF-κB/Rel family genes was relatively up-regulated in the former (39).

The relatively delayed IκBα degradation and RelA nuclear translocation in LPS-stimulated BMM cells is reminiscent of the phenotypes of tumor-associated macrophages and MyD88-deficient macrophages (40, 41), both of which are relatively poor producers of proinflammatory cytokines. BMM also showed relatively prolonged formation of a NF-κB DNA binding complex most likely corresponding to p50 homodimers. p50 lacks a transactivation domain (42). In this context, TNF-α mRNA levels, which were significantly lower in LPS-stimulated BMM than in LPS-stimulated GM-BMM, are thought to be reduced by p50 binding to κB elements in the TNF-α promoter (43, 44). Furthermore, p50 has recently been shown to promote the transcription of IL-10 (45) and we found here that IL-10 mRNA levels were significantly up-regulated in BMM compared with GM-BMM following LPS treatment. Interestingly, in that prior study (45) it was shown that macrophages generated from p50-deficient mice exhibited a skewed cytokine response to LPS characterized by decreased IL-10 and increased TNF-α and IL-12 production and that RelA and c-*rel* primarily regulated the expression of TNF-α and IL-12 but played little or no role in driving IL-10 expression (45). Consistent with this observation, IL-10 expression was found to relate to high levels of p50 expression (46), and the inhibition of RelA-containing complexes up-regulated IL-10 production (47). Even though further analysis is obviously warranted, it would appear that our findings are at least consistent with these studies linking NF-κB DNA binding activity and relative cytokine expression.

GM-BMM cells displayed faster and enhanced AP-1 activation in response to LPS than did BMM cells. NF-κB regulates the expression of AP-1 family genes (48) and there appears to be significant crosstalk between the two pathways (49). Because AP-1 activity appears to play a role in the regulation of IL-12 (50) and TNF-α (51), for example, our findings on the relative AP-1 activities of stimulated GM-BMM and BMM could also help to explain the relative cytokine profiles.

Given that GM-BMM cells display a proinflammatory profile upon LPS stimulation and that GM-CSF has been reported to have many of the same "priming" properties as IFN-γ on macrophages (34, 36), we therefore explored the effects of GM-CSF on the cytokine profile of BMM. The addition of GM-CSF to BMM cultures of itself did not lead to detectable cytokine production. However, with this "priming" protocol the LPS-stimulated BMM cells were now capable of producing IL-12p70 and IL-23 as well as more TNF-α. In other words, the BMM cells could be altered to adopt to some extent the pattern of the GM-BMM cell response. "Priming" of BMM with GM-CSF resulted in increased RelA nuclear translocation and the formation of a RelA-containing NF-κB DNA binding complex, possibly helping to explain some of these cytokine changes. Likewise, GM-BMM, when treated with M-CSF, could also adopt the BMM phenotype to some degree by producing less IL-12p70, IL-23, and TNF-α but more CCL2 following LPS stimulation. This M-CSF pretreatment resulted in the loss of the RelA-containing NF-κB DNA binding complex, which again may help explain the observed changes in cytokine production. In contrast, M-CSF enhanced the production of CCL2 but had no effect on LPS-induced IL-10 production from GM-BMM cells. Taken together, these data show that cytokine production joins the list of responses where GM-CSF and M-CSF can have opposing actions on macrophage populations (20, 21).

Several of our findings point to a clear "proinflammatory" function for GM-CSF on macrophage populations, consistent with it acting at sites of inflammation where its levels are elevated above the normally low steady-state levels (34). In vivo it is likely that macrophage populations will be normally exposed to the ubiquitously expressed M-CSF, thereby controlling their numbers in many tissues (9, 32). BMM cells preferentially expressed IL-10 and, when added to GM-BMM, M-CSF suppressed the elevated TNF-α, IL-12p70, and IL-23 production; these latter responses of macrophages to M-CSF, as well as others (20, 21), indicate that it may dampen inflammation in certain circumstances. Perhaps part of its homeostatic role in the steady state is to provide "protection" from or "resistance" to inappropriate proinflammatory signals being imparted to the macrophage. The influence that each CSF has on the expression of components of the NF-κB/Rel and AP-1 signaling pathway is currently under investigation and may help our understanding of the role of these CSFs in macrophage-dependent inflammatory responses.

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Disclosures

The authors have no financial conflict of interest.

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